

## Research article

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**p14<sup>ARF</sup> expression in invasive breast cancers and ductal carcinoma *in situ* – relationships to p53 and Hdm2**SB Vestey<sup>1</sup>, C Sen<sup>2</sup>, CJ Calder<sup>2</sup>, CM Perks<sup>1</sup>, M Pignatelli<sup>2</sup> and ZE Winters<sup>1</sup><sup>1</sup>University of Bristol Division of Surgery, Bristol Royal Infirmary, Bristol, UK<sup>2</sup>Department of Histopathology, United Bristol Healthcare NHS Trust, Bristol Royal Infirmary, Bristol, UKCorresponding author: ZE Winters, [zoe.winters@bristol.ac.uk](mailto:zoe.winters@bristol.ac.uk)

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*Breast Cancer Res* 2004, **6**:R571-R585 (DOI 10.1186/bcr912)© 2004 Vestey *et al.*; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.**Abstract**

**Introduction** p14<sup>ARF</sup> stabilises nuclear p53, with a variable expression of p14<sup>ARF</sup> mRNA in breast cancers. *In vitro*, nuclear p14<sup>ARF</sup> binds Hdm2 to block Hdm2-dependent nucleocytoplasmic shuttling of p53, which is required before cytoplasmic degradation of p53. p14<sup>ARF</sup> is negatively regulated by p53 and through p53-independent pathways. No studies have yet examined levels of p14<sup>ARF</sup> protein expression in breast cancer and their relationship to Hdm2/p53 immunoreactivity or subcellular localisation. Previously, immunohistochemical expression of cytoplasmic p14<sup>ARF</sup>, p53 and Hdm2 has been described. HER-2 (*c-erbB2/neu*) predicts prognosis and interacts with the p14<sup>ARF</sup>/Hdm2 pathway to inactivate p14<sup>ARF</sup> and to influence Hdm2 activity and localisation. This study examined p14<sup>ARF</sup> and p53/Hdm2 expression and subcellular localisation by using immunohistochemistry in a series of invasive ductal breast cancers (IDCs) with concomitant ductal carcinoma *in situ* (DCIS), to evaluate whether findings *in vitro* were related to clinicopathological parameters such as HER-2 and their effect on patient outcome.

**Methods** The 4C6 anti-p14<sup>ARF</sup> monoclonal antibody and Dako Envision Plus system were used to evaluate p14<sup>ARF</sup> expression in 103 patients; p53/Hdm2 staining was performed.

**Results** p14<sup>ARF</sup> was evaluable in 96 patients, with nuclear p14<sup>ARF</sup> expression (modified Quick-score  $\geq 3$ ) in 79% ( $n = 76$ )

of IDCs and in associated DCIS in 74 patients. Cytoplasmic p14<sup>ARF</sup> was detectable in 23 breast cancers. Nuclear and cytoplasmic p14<sup>ARF</sup> showed no correlation with p53 subcellular immunoreactivity. Increasing levels of cytoplasmic p14<sup>ARF</sup> were associated with nuclear and cytoplasmic Hdm2 expression ( $P < 0.001$ ). Subcellular ARF expression was not associated with clinicopathological parameters, and although not an independent prognosticator, these preliminary findings suggest that cytoplasmic p14<sup>ARF</sup> might be associated with a better overall survival ( $P = 0.09$ ; log rank). The association between HER-2 positivity and nuclear p14<sup>ARF</sup> ( $P = 0.038$ ), as well as nuclear Hdm2 ( $P = 0.019$ ), reflects the *in vitro* findings of HER-2 interaction with the ARF/Hdm2 pathway. Cytoplasmic p53 and Hdm2 expression might have biological implications, through an association of cytoplasmic p53 with increased tumour proliferation ( $P = 0.005$ ), and an improved overall survival ( $P = 0.002$ , log rank) in cytoplasmic Hdm2-expressing tumours, that independently predict favourable overall survival ( $P = 0.02$ ) and disease-free survival ( $P = 0.03$ ).

**Conclusions** Nuclear p14<sup>ARF</sup> expression is similar in IDCs and DCIS and is associated with Hdm2 immunoreactivity. Nuclear p14<sup>ARF</sup> and Hdm2 might be regulated by HER-2. Clearly, our findings *in vivo* suggest a complexity of p14<sup>ARF</sup>/Hdm2 and p53 pathways in which consideration of cytoplasmic p14<sup>ARF</sup> and Hdm2 might have tumorigenic implications.

**Keywords:** ARF protein, ductal carcinoma *in situ*, Hdm2, HER-2, immunohistochemistry

**Introduction**

The development and growth of breast cancers result from the inactivation of p53 or retinoblastoma (pRb) tumour suppressor proteins that regulate cell cycle control. Such regulatory pathways trigger cell cycle arrest or apoptosis in response to intracellular challenges such as DNA damage,

hypoxia and oncogene activation, with preservation of genomic stability [1]. pRb maintains checkpoint integrity through binding and blocking E2F transcription factors, a process reinforced by the G1 cyclin-dependent kinase inhibitor p16<sup>INK4a</sup> [2]. Similarly, p53 accumulates to transcriptionally activate the cyclin-dependent kinase inhibitor

cc = Spearman correlation coefficient; DCIS = ductal carcinoma *in situ*; DFS = disease-free survival; ER = oestrogen receptor; GPG = good prognostic group; HRP = horseradish peroxidase; IDC = invasive ductal cancer; MPG = moderate prognostic group; MQS = modified Quick-score; NPI = Nottingham Prognostic Index; OS = overall survival; PPG = poor prognostic group; pRb = retinoblastoma protein; TBS = Tris-buffered saline.

p21<sup>WAF1/CIP1</sup>, as well as its own negative regulator Hdm2 (human Mdm2; murine double minute 2), which terminates the p53 response. Nuclear retention of p53 underlines its tumour suppressor response and suggests an additional pathway for disabling p53 over and above its mutation in more than 50% of human cancers [3-5]. In an autoregulatory feedback loop, Hdm2 maintains low levels of p53 in normal non-stressed cells and inhibits nuclear p53 through multiple and diverse mechanisms. Hdm2 binds p53 to inhibit its transactivation function and shuttles p53 from the nucleus to the cytoplasm to facilitate its degradation [6-8]. Hdm2 is also an E3 ubiquitin ligase that targets p53 for the ubiquitin-dependent 26S proteasome in the cytoplasm [9].

The INK4a/ARF gene locus on chromosome 9p21 encodes p16<sup>INK4a</sup> and p14<sup>ARF</sup>, both of which act in tumour surveillance and link the pRb and p53 pathways [2,10-14]. p14<sup>ARF</sup> is encoded from an alternative, but partly overlapping, reading frame together with p16<sup>INK4a</sup> [15], such that ARF has a separate first exon (1 $\beta$ ) that splices into common exons 2 and 3, shared with p16<sup>INK4a</sup>. These proteins independently target two cell cycle control pathways, with p16<sup>INK4a</sup> inhibiting cyclin D1/cyclin-dependent kinases within the pRb pathway and p14<sup>ARF</sup> inhibiting the oncoprotein Hdm2 within the p53 pathway [10-14]. ARF expression and activation occur through mitogenic signals such as Myc, E1A, E2F1, Ras and v-Abl, stabilising p53, followed by cell cycle arrest or apoptosis [16,17]. Consequently, ARF connects the pRb and p53 pathways, with excessive proliferative signalling via pRb activating arrest mechanisms by p53. This suggests that loss of ARF function is a major contributor to carcinogenesis in humans [17]. p14<sup>ARF</sup> is a true tumour suppressor protein; tumours develop spontaneously in *ARF*-null mice [14,18]. Promoter hypermethylation of the *INK4a* and *ARF* genes is a major mechanism of their inactivation, followed by hemizygous deletions [2,10,11,19]. Breast cancers rarely demonstrate homozygous deletions of either gene, with no mutations of *ARF* [2,19-21].

ARF expression *in vitro* and *in vivo* is associated with a G1 or G2 cell cycle arrest or apoptosis after inappropriate mitogenic stimuli or DNA damage [10-13,22,23]. Although these effects in large part relate to activation of the p53 pathway, recent reports suggest that p14<sup>ARF</sup> inhibits growth independently of p53 [12,17,23,24]. Low or undetectable p14<sup>ARF</sup> expression in normal tissues and its function independently of p53 in highly proliferating, homeostatic tissues, in comparison with tumours, suggests that p14<sup>ARF</sup> function has yet to be completely established [17,24]. Subcellular localisation of p14<sup>ARF</sup> is preferentially nucleolar or nuclear, where it binds Hdm2 to inhibit the latter's activities towards p53 [15,25-27]. ARF-bound Hdm2 blocks Hdm2-dependent nucleocytoplasmic shuttling of p53, to produce nuclear retention and activation of p53

[13,27]. Further, ARF averts degradation of p53 by inhibiting Hdm2-dependent ubiquitination of p53 *in vivo* [13,28,29]. p14<sup>ARF</sup> acts upstream of p53 and is subject to its negative feedback regulation, suggesting that p53 mutations or its inactivation by *HDM2* amplification are often accompanied by overexpression of ARF [10-14,30]. p53-positive tumours are also likely to have sustained epistatic mutations such as *HDM2* amplification or *ARF* loss [11]. Inactivation of ARF might occur through the overexpression of several ARF repressors, including Twist and Tbx-2 in breast cancers and Bmi-1 in other tumours [31,32].

Nuclear import and export is a feature of both p53 and Hdm2 with implications for their functional regulation, such that cytoplasmic p53 is associated with tumours with a poor prognosis [3-6,8]. Just as Hdm2 regulates the nuclear export of p53, *in vivo* evidence suggests that ARF influences the subcellular localisation of Hdm2 [21]. Cytoplasmic ARF occurs through binding to Pex19p with evidence of weak to moderate cytoplasmic staining in human tumours, whereas two studies disregarded cytoplasmic p14<sup>ARF</sup> expression [33-37]. In a single *in vitro* study, mouse p19<sup>ARF</sup> binds a cytoplasmic protein *Pex19p* (cloned from mouse testis) in normal cells (NIH 3T3) [33]. Consequently, the subcellular localisation of these proteins and the relationship between their levels of expression are likely to be important in evaluating breast cancers and their development from pre-invasive ductal carcinoma *in situ* (DCIS) to invasive disease.

Previous studies have examined p14<sup>ARF</sup> mRNA expression in breast cancers, with evidence suggesting altered expression and an association with p53 [38,39]. The aim of this study was to evaluate the levels of protein expression for p14<sup>ARF</sup> in relation to Hdm2 and p53 using immunohistochemistry in DCIS and invasive ductal breast cancers (IDCs), including the study of their subcellular localisation. So far, no studies have examined p14<sup>ARF</sup>/Hdm2 and p53 in relation to clinicopathological parameters and prognosis in breast cancer. We have shown nuclear p14<sup>ARF</sup> expression in 79% of IDCs and in associated DCIS in 74 patients. Cytoplasmic p14<sup>ARF</sup> was detectable in 23 breast cancers. Levels of expression and subcellular localisation for p14<sup>ARF</sup>, Hdm2 and p53 were similar in IDCs and DCIS. ARF expression showed no correlation with p53 immunoreactivity and was associated with Hdm2 nuclear and cytoplasmic expression. HER-2-positive breast cancers were associated with nuclear p14<sup>ARF</sup> and nuclear Hdm2 immunoreactivity. Our preliminary findings suggest that cytoplasmic p14<sup>ARF</sup> and cytoplasmic Hdm2-expressing breast cancers might be associated with a better outcome.

**Table 1****Clinicopathological variables in the patients entered into this study**

Characteristic	No. of patients (%)
Total	103 (100)
Age (years)	
≤50	31 (30)
>50	72 (70)
Invasive tumours	
Lymph node status	
Negative	37 (41)
Positive	53 (59)
Not assessed	13
Invasive tumour grade	
Grade I	16 (16)
Grade II	51 (49)
Grade III	36 (35)
Invasive tumour size (cm)	
≤2 cm	53 (52)
>2 cm	48 (48)
Multifocal	2
Lymphovascular invasion	
Present	50 (50)
Absent	50 (50)
Not assessed	3
NPI	
GPG < 3.4	34 (39)
MPG 3.4–5.4	37 (42)
PPG > 5.4	17 (19)
Not possible to calculate	15
ER (quick-score)	
Positive (4–8)	63 (64)
Negative (0–3)	36 (36)
Not assessed	4
HER-2 IHC	
Negative (0/1+)	87 (84)
Positive (2+/3+)	16 (16)
Ki67 IHC	
Low proliferation, <10%	51 (50)
High proliferation, ≥10%	52 (50)
Ductal carcinoma <i>in situ</i>	
VNPC	

**Table 1 (Continued)****Clinicopathological variables in the patients entered into this study**

Grade I	23 (24)
Grade II	36 (37)
Grade III	38 (39)
Not assessed	6
ER (quick-score)	
Positive (4–8)	61 (64)
Negative (0–3)	35 (36)
Not assessed	7
HER-2 IHC	
Negative (0/1+)	77 (80)
Positive (2+/3+)	19 (20)
Not assessed	7
Ki67 IHC	
Low proliferation, <10%	61 (59)
High proliferation, ≥10%	42 (41)

ER, oestrogen receptor; GPG, good prognostic group; IHC, immunohistochemistry; MPG, moderate prognostic group; NPI, Nottingham Prognostic Index; PPG, poor prognostic group; VNPC, Van Nuys Pathologic classification.

**Methods****Patients**

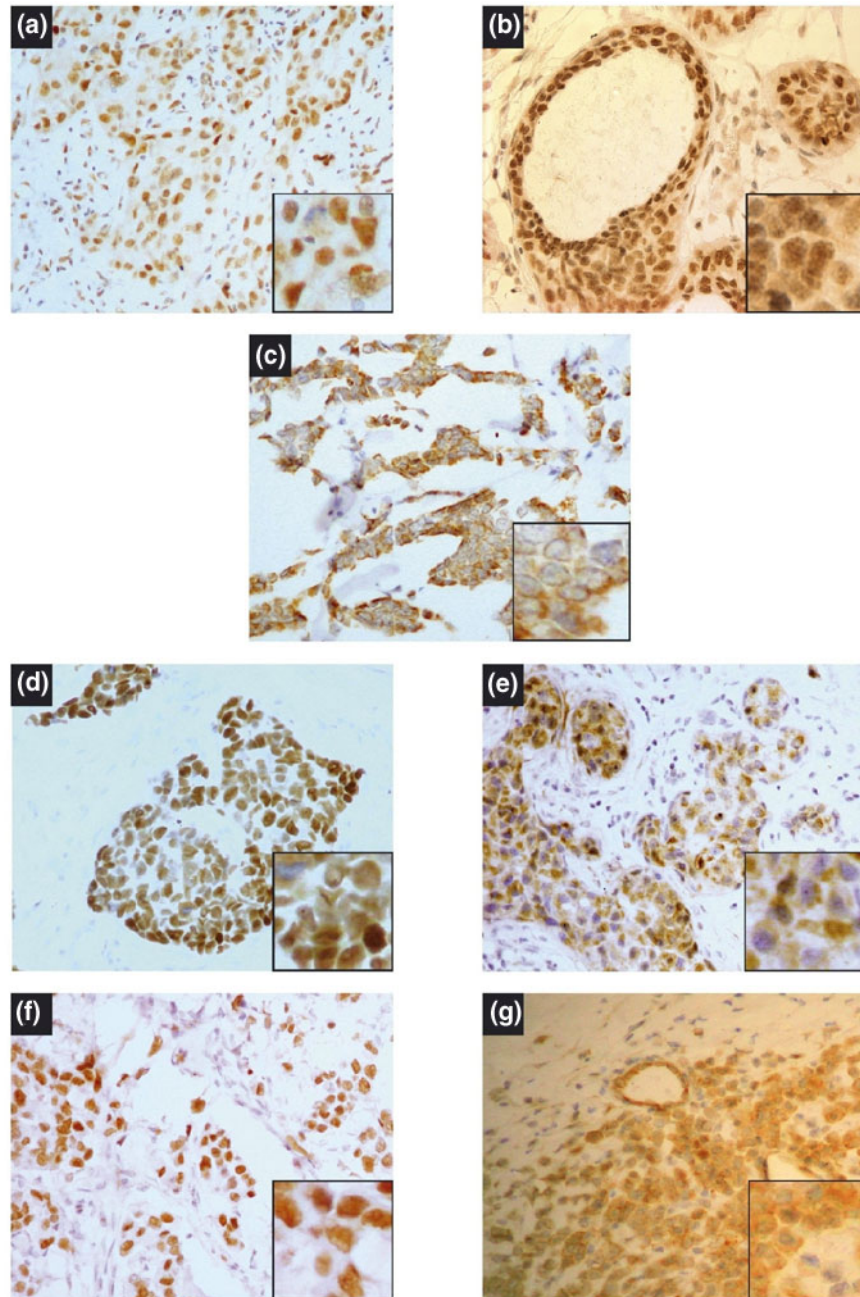
The study included 103 patients aged 26–88 years (median 59 years) with IDC of the breast, in association with concomitant DCIS diagnosed between 1996 and 2000 at the Bristol Royal Infirmary, Bristol, UK (Table 1). Eligibility criteria were histological diagnosis of IDC and DCIS, no distant metastases, and unilateral tumour. Regional Ethics Committee approval was granted before commencement of the study. Axillary lymphadenopathy was evaluable in 90 patients: 37 (41%) of the patients were lymph node negative, and 53 (59%) were node positive (N1 [mobile ipsilateral] or N2 [fixed ipsilateral]). No axillary surgery was undertaken in the remaining 13 patients because of age-related co-morbidity.

Clinicopathological subgroups were analysed in accordance with the Nottingham Prognostic Index (NPI) and divided into good (GPG), moderate (MPG) and poor (PPG) prognostic groups as described, with a modification that included no assessment of the internal mammary lymph nodes [40]. Evaluation of the NPI was precluded in 15 patients because of non-evaluable regional lymphadenopathy and tumour size. Similarly, subgroups of DCIS were analysed in accordance with the Van Nuys Pathologic Classification (Table 1), which was not assessable in six patients [41]. The design of the study to include representative samples of synchronous IDC and DCIS precluded analysis by the Van Nuys Prognostic Index [41]. Adjuvant treatment groups comprised tamoxifen in 60 patients (27

GPG, 16 MPG, 4 PPG and 13 no NPI), and cyclophosphamide–methotrexate–5-fluorouracil-containing and anthracycline-containing regimes in 17 and 21 patients, respectively (4 GPG, 19 MPG, 13 PPG and 2 no NPI). Five patients received no adjuvant treatment. The median follow-up duration was 51 months (range 6–120 months). All were primary tumours with the exception of six local tumour recurrences, which were excluded from the analysis of patient outcome (see Table 4 and Fig. 3).

Tumour samples were collected shortly after surgery and were fixed in buffered formalin for 24–48 hours at room temperature (20–22°C). Tumours were classified in accordance with the guidelines of the UK National Health Service Breast Screening Programme [42] and were graded by the modified Bloom's grading system described by Elston and Ellis [43]. Oestrogen receptor (ER) immunostaining was performed with a standard three-layered streptavidin–avidin–biotin horseradish peroxidase (HRP) method with a mouse anti-human ER primary antibody (M0747, 1:100 dilution; Dako, Ely, UK) and a biotinylated rabbit anti-mouse secondary antibody (E0354 1:350 dilution; Dako). Expression of ER was assessed with Quick-score (0–8) and classified as positive (4–8; more than 3) or negative (0–3; 3 or less) in five high-power fields [44].

Tumour proliferation was assessed with nuclear Ki67 immunostaining (polyclonal rabbit anti-human Ki67 antigen; A0047, 1:100 dilution; Dako). A goat anti-rabbit biotin-labelled polypeptide (E432, 1:400 dilution; Dako) was

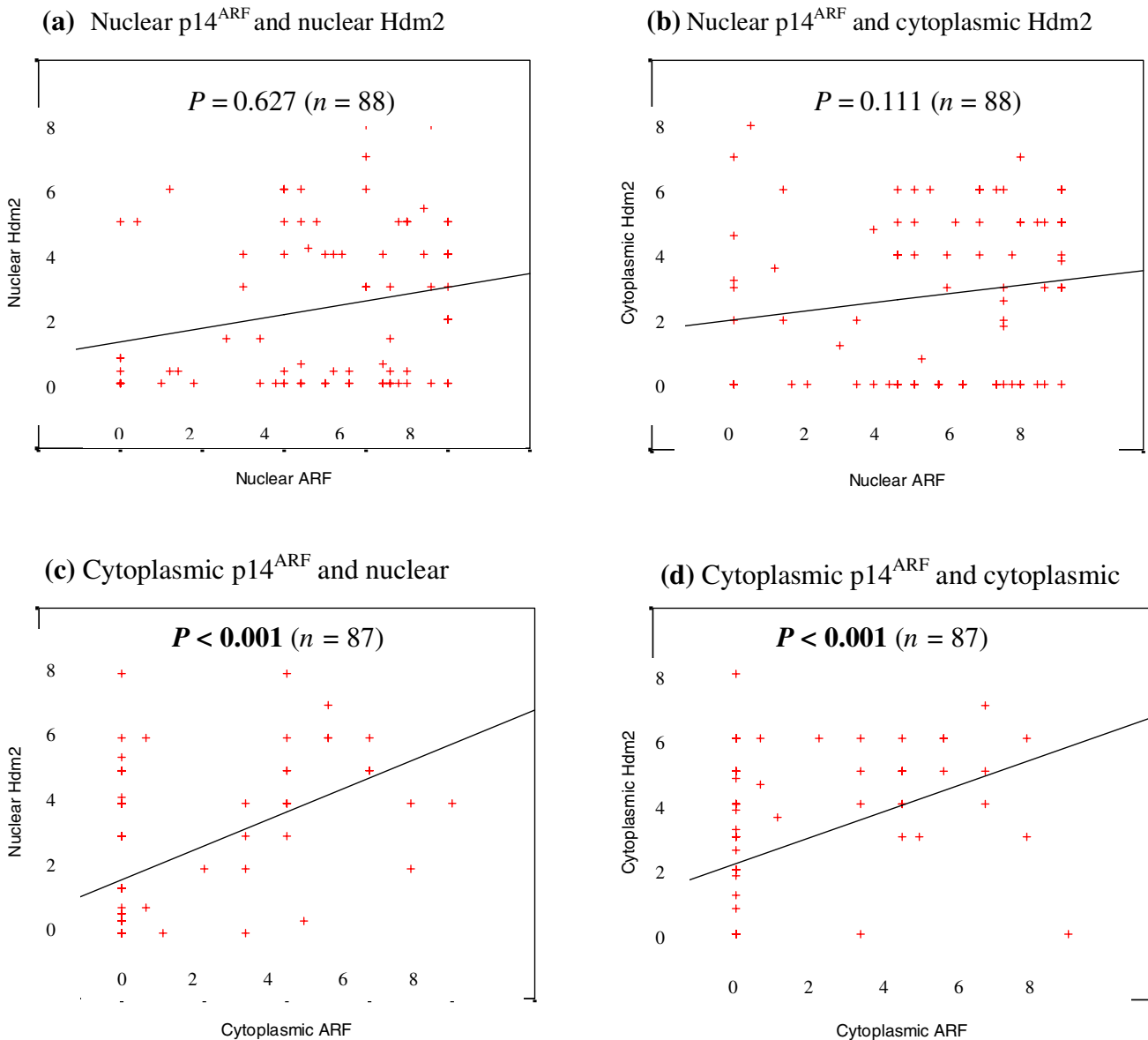
**Figure 1**

Examples of p14<sup>ARF</sup>, p53 and Hdm2 immunoreactivity in infiltrating ductal carcinoma of the breast. Immunostaining was performed as described in the Methods section, and nuclei were counterstained with haematoxylin. **(a)** A tumour showing nuclear p14<sup>ARF</sup> expression in invasive ductal cancer; **(b)** a similar nuclear p14<sup>ARF</sup> positivity in concomitant ductal carcinoma *in situ* (DCIS). **(c)** Cytoplasmic p14<sup>ARF</sup> expression in invasive ductal cancer is representative of similar expression in DCIS. Representative examples of expression of nuclear p53 **(d)**, cytoplasmic p53 **(e)**, and also nuclear Hdm2 **(f)** and cytoplasmic Hdm2 **(g)**. High-power magnification (×400) (a-g). Insets show higher-power (×1000) views of the same fields.

used as a secondary antibody. Tonsillar tissue was used as a positive control and primary antibody was replaced with Tris-buffered saline (TBS) as a negative control. Ki67 staining was evaluated as positive or negative, with low proliferation indicative of less than 10% of positive-staining cells, compared with high proliferation with at least 10% positiv-

ity [45]. HER-2 immunostaining was performed with the mouse monoclonal anti-HER-2 antibody (RTU-CB11; NovaCastra/Vector, Newcastle upon Tyne, UK) and the Envision Plus HRP system (K4006; Dako). HER-2 expression was scored according to the degree and proportion of membrane staining, with a score of 0 or 1+ defined as neg-

Figure 2



Relationships between subcellular localisation of p14<sup>ARF</sup> and Hdm2. Scatter plots according to nuclear p14<sup>ARF</sup> modified Quick-score (MQS) (a, b) and cytoplasmic p14<sup>ARF</sup> MQS (c, d). p14<sup>ARF</sup> and Hdm2 staining was analysed as described in the Methods section. (a, b) Distribution of nuclear p14<sup>ARF</sup> and nuclear Hdm2 (a), and also cytoplasmic Hdm2 (b), showed no significant correlations. (c, d) Distribution of cytoplasmic p14<sup>ARF</sup> and nuclear Hdm2 (c), and also cytoplasmic Hdm2 (d), showed significant correlations ( $P < 0.001$ , respectively).  $P$  values in bold ( $P < 0.05$ ) indicate significance.

ative, and 2+ or 3+ as HER-2 positive [46]. Lymphovascular invasion was assessed as present or not, and was analysed together with ER, HER-2 and Ki67 in the Department of Pathology (by CS and CJC).

**Immunohistochemistry**

p14<sup>ARF</sup> immunoreactivity was evaluated with the mouse monoclonal antibody 4C6 (kindly provided by Dr Gordon Peters, Cancer Research UK, Lincoln's Inn Fields, London,

UK) at a 1:200 dilution of 15 µg/ml. ARF immunostaining of IDC and DCIS was compared with formalin-fixed paraffin-embedded cell buttons, using H1299 non-small cell lung cancer cells as a positive control for p14<sup>ARF</sup>, and MCF-7 breast cancer cells as a negative control. Further negative controls replaced 4C6 with sero-matched IgG2a used on previously established p14<sup>ARF</sup>-expressing breast cancers. The 4C6 antibody with epitope recognition of carboxy-terminal p14<sup>ARF</sup> was chosen after testing of other

mouse monoclonal antibodies including C-18 (Santa Cruz Biotechnology, California) and 14PO2 (LabVision/Neomarkers, Fremont, California). The specificity of 4C6 has been demonstrated previously [13,27].

Hdm2 expression was assessed with the mouse monoclonal antibody OP46 (Oncogene Research, CN Biosciences, Nottingham, UK) that detects C-terminal Hdm2 with specificity for the 90 kDa isoform [47]. OP46 was selected after testing with other antibodies including 2A10 and 3G5 (kindly given by AJ Levine, Rockefeller University, New York) both of which had technical or other limitations [48]. OP46 was used at a 1:80 dilution of 100 µg/ml and evaluated in the context of Hdm2 overexpressing A375 malignant melanoma cells, and negatively controlled with IgG2b, which was substituted for the primary antibody. Biotinylated rabbit anti-mouse IgG secondary antibody (E0354, 1:300 dilution of 1.26 mg/ml; Dako), together with the Strept-AB Complex/HRP (0377; Dako, Glostrup, Denmark), was used for p14<sup>ARF</sup> and Hdm2. Technical limitations precluded dual staining for ARF and Hdm2. p53 immunostaining was performed as described [49] by using the DO7 monoclonal antibody (NovaCastra/Vector). Breast carcinomas known to overexpress p53 with known TP53 gene mutations and protein accumulation were used as positive controls. Negative controls were obtained by omitting the primary antibodies.

Formalin-fixed paraffin sections of breast cancer tissue and cell pellets (controls) were mounted on 3-aminopropyl-triethoxysilane-coated (Sigma, Poole, Dorset, UK) glass slides and were baked for 30 min at 56–60°C before being dewaxed in Clearene (Surgipath Europe, Peterborough, UK). The tissue was rehydrated by sequential immersion in 100% and 50% ethanol to distilled water. Tissue sections were subjected to heat retrieval of antigens in citrate buffer (pH 6) in a pressure cooker for 3 min; after cooling they were incubated in 0.3% (v/v) hydrogen peroxide for 5 min. Subsequently, sections were washed in tapwater and TBS (pH 7.45). Before incubation with p14<sup>ARF</sup> and Hdm2 primary antibodies, sections were exposed to avidin and biotin blocking solutions (Vector Laboratories, Burlingame, California) for 15 min each. Further blocking was achieved through exposure to normal rabbit serum (diluted with TBS) for 30 min at room temperature. Primary antibody was applied and incubated at 4°C overnight (18 hours) for p14<sup>ARF</sup> and Hdm2, except for p53, which was incubated for 1 hour at room temperature. After washing with TBS, biotinylated rabbit anti-mouse secondary antibody was applied for 30 min at room temperature. The Strept-AB Complex/HRP was used for the detection of p14<sup>ARF</sup> and Hdm2, and involved an additional 30 min incubation with TBS/biotinylated streptavidin/HRP. Staining was revealed by development in the chromogen 3,3-diaminobenzidine

tetrahydrochloride for 5–10 min before counterstaining with haematoxylin before mounting.

Immunostaining was assessed with a Zeiss Axioskop microscope with a 40 × Achrostig mat lens (×400 overall magnification) and a field diameter of 0.46 mm. In the neoplastic cell population for IDC and DCIS, the degree of staining intensity and the proportion of cells with p14<sup>ARF</sup>, Hdm2 and p53 immunoreactivity in the nucleus and cytoplasm were individually graded semiquantitatively to produce an intensity distribution score or *H*-score for each localisation, with invasive and pre-invasive components given separate scores [50]. Initial scoring was of 10 high-power fields; however, in view of the homogeneous staining this was reduced to 5 high-power fields. A modified Quick-score (MQS) was used to define a score of 0–3 for intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and 0–5 for distribution (0, no staining; 1, 1% or less; 2, 1–10%; 3, 11–33%; 4, 34–66%; 5, 67–100% of cells staining), giving an overall score of 0–8 [44]. Sections were scored independently by two pathologists (CS and CJC) in the Department of Pathology. Unless otherwise stated, scores were assessed as a continuum for the purposes of statistical correlation. For the purposes of describing p14<sup>ARF</sup>, Hdm2 and p53 immunoreactivity, tumour positivity was defined as a modified Quick-score of 3 or more (1+/2+) (see Table 2).

### Statistical analysis

Data were analysed with the SPSS 10.0 for Windows (SPSS Incorporated, Chicago, Illinois) statistics software and summarised with descriptive statistics. The associations between p14<sup>ARF</sup>, p53 and Hdm2 and patient characteristics were assessed with the Spearman non-parametric test for continuous variables and the  $\chi^2$  test and Fisher's exact test for categorical factors. The relationship between p14<sup>ARF</sup> localisation and the subcellular localisation of p53 and Hdm2 was analysed with the Spearman correlation coefficient (cc) for non-parametric data, with the p14<sup>ARF</sup> and p53/Hdm2 MQS used as continuous variables. Analyses of survival data were performed with the log-rank test and the Cox regression model, with survival curves computed with the Kaplan–Meier method. For p14<sup>ARF</sup>, p53 and Hdm2, univariate and multivariate analyses were performed and adjusted for the NPI score and treatment received (tamoxifen/chemotherapy/none). Because the NPI is based on nodal involvement, on tumour size and on grade, patients ( $n = 11$ ) with non-evaluable lymphadenopathy and tumour size were excluded from the multivariate regression analyses (see Table 4).

**Table 2****Immunohistochemical MQSs (0–8) for p14<sup>ARF</sup>, p53 and Hdm2 expression in the nucleus and cytoplasm of invasive ductal cancer and ductal carcinoma in situ**

Protein	Cancer type	Location	Tumour no. (%)	MQS 0–2 (0)	MQS 3–5 (1+)	MQS 6–8 (2+)
p14 <sup>ARF</sup>	Invasive	Nucleus	96	20 (21)	31 (32)	45 (47)
		Cytoplasm	95	72 (76)	16 (17)	7 (7)
	DCIS	Nucleus	91	17 (17)	29 (32)	45 (49)
		Cytoplasm	91	70 (80)	13 (14)	8 (9)
p53	Invasive	Nucleus	93	62 (67)	17 (18)	14 (15)
		Cytoplasm	93	70 (75)	18 (19)	5 (5)
	DCIS	Nucleus	91	66 (73)	12 (13)	13 (14)
		Cytoplasm	91	58 (62)	27 (29)	6 (6)
Hdm2	Invasive	Nucleus	92	49 (53)	33 (36)	10 (11)
		Cytoplasm	91	42 (46)	33 (36)	16 (18)
	DCIS	Nucleus	91	51 (56)	25 (27)	15 (16)
		Cytoplasm	91	43 (47)	27 (30)	21 (23)

Tumour numbers scored (MQS 0–8) by immunohistochemistry for p14<sup>ARF</sup>, p53 and Hdm2 expression in the nucleus and cytoplasm of invasive cancers and ductal carcinoma *in situ* (DCIS) are shown. Immunohistochemistry expression was assessed individually in the nucleus and the cytoplasm, and is defined as negative (0) (MQS 0–2), weak/moderate (1+) (MQS 3–5) and strong (2+) (MQS 6–8). Positivity for p14<sup>ARF</sup>, p53 and Hdm2 expression was defined as an MQS of 3 or more (1+/2+).

**Results****p14<sup>ARF</sup> levels of expression and subcellular localisation in IDC and DCIS**

p14<sup>ARF</sup> immunoreactivity was evaluable in the nucleus and cytoplasm in 96 (93%) cases of IDC and in associated DCIS in 91 (88%) cases (Fig. 1 and Table 2). ARF expression (MQS  $\geq$  3) was detectable in 76 (79%) IDCs and was predominantly nuclear, with 23 breast cancers (24%) demonstrating cytoplasmic p14<sup>ARF</sup>. Nuclear ARF was homogeneous, with no distinct pattern of nucleolar staining or exclusive nucleolar p14<sup>ARF</sup> expression [30,34,35].

Predominant nuclear ARF might have been 'masking' the accurate delineation of nucleolar p14<sup>ARF</sup>. Weak/moderate nuclear ARF expression (MQS 3–5) was evident in 31 (32%) cases, with half of these showing cytoplasmic ARF ( $n = 16$ ). A loss of p14<sup>ARF</sup> nuclear immunostaining was observed in 20 (21%) invasive breast cancers, whereas the remaining 45 (47%) cases exhibited diffuse and strong nuclear staining (MQS 6–8; Table 2), with a similar intensity of cytoplasmic ARF in 7 cases only. A comparison showed that the levels of p14<sup>ARF</sup> expression in DCIS were similar to invasive disease (Table 2).

**Relationships between p14<sup>ARF</sup> and p53 levels of expression and subcellular localisation in IDC and DCIS**

p53 immunoreactivity and subcellular localisation were evaluable in 93 (90%) cases of IDC and in DCIS in 91 (88%) cases. Immunohistochemically, p53 was detectable in 31 (33%) IDCs (MQS  $\geq$  3), with similar levels of nuclear

(31 cases) and cytoplasmic (23 cases) expression (25%), compared with p14<sup>ARF</sup> (Table 2). Weak to moderate (MQS 3–5) nuclear p53 expression was observed in 17 cases of IDC (18%), with similar cytoplasmic staining (18 cases). There was no nuclear or cytoplasmic p53 expression (MQS 0–2) in most IDCs (67% and 75%, respectively). Strong nuclear p53 expression (MQS 6–8) was evident in 15% of IDCs, with 5% of these expressing intense cytoplasmic staining. A comparison of the levels of p53 expression and subcellular localisation in DCIS were similar to those in IDC (Table 2).

Sixty-six percent ( $n = 46$ ) of nuclear p14<sup>ARF</sup>-expressing breast cancers (1+/2+) were associated with absent p53 nuclear immunoreactivity, or the possibility of wild-type p53 (Table 3). By comparison, proportionately fewer or one-third of ARF-expressing tumours ( $n = 24$ ) had nuclear p53 expression (1+/2+). These associations did not reach statistical significance. Nuclear p14<sup>ARF</sup> expression (1+/2+) showed no particular pattern of p53 subcellular immunoreactivity (data not shown).

**Relationships between p14<sup>ARF</sup> and Hdm2 levels of expression and subcellular localisation in IDC and DCIS**

Hdm2 expression and subcellular distribution were evaluable in 92 (89%) cases of IDC and in 91 cases of DCIS (Fig. 1 and Table 2). Nuclear Hdm2 was detectable (MQS  $\geq$  3) in 43 (47%) IDCs with a similar proportion (49 cases) of cytoplasmic staining (Table 2). A minority of IDCs strongly expressed nuclear and cytoplasmic Hdm2 (MQS 6–8) in



**Table 3****Relationships between nuclear expression of p14<sup>ARF</sup> with nuclear p53 and Hdm2 in invasive breast cancers**

		Nuclear p14 <sup>ARF</sup> MQS		
		0 (n = 19)	1+ (n = 28)	2+ (n = 42)
Nuclear p53 MQS	0 (n = 59)	13	21	25
	1+ (n = 17)	2	5	10
	2+ (n = 13)	4	2	7
		Nuclear p14 <sup>ARF</sup> MQS		
		0 (n = 16)	1+ (n = 31)	2+ (n = 41)
Nuclear Hdm2 MQS	0 (n = 48)	13	17	18
	1+ (n = 31)	2	10	19
	2+ (n = 9)	1	4	4
		Nuclear p53 MQS		
		0 (n = 58)	1+ (n = 15)	2+ (n = 11)
Nuclear Hdm2 MQS	0 (n = 47)	35	6	6
	1+ (n = 34)	23	7	4
	2+ (n = 3)	0	2	1

Tumour numbers scored (MQS 0–8) by immunohistochemistry for nuclear p14<sup>ARF</sup> are compared with those for nuclear p53; nuclear p14<sup>ARF</sup> with nuclear Hdm2; and nuclear p53 with nuclear Hdm2. Nuclear expression of p14<sup>ARF</sup>, Hdm2 and p53 is represented as negative (0) (MQS 0–2), weak/moderate (1+) (MQS 3–5) and strong (2+) (MQS 6–8). Tumour numbers reflect the combined assessment of protein pairs. Positivity for p14<sup>ARF</sup>, p53 and Hdm2 expression was defined as 1+/2+ (MQS ≥ 3).

10 (11%) and 16 (18%) tumours, respectively. About half of invasive cancers were negative for nuclear (53%) and cytoplasmic (46%) Hdm2. A similar pattern for all levels of Hdm2 expression was seen in DCIS.

Proportional numbers of nuclear p14<sup>ARF</sup>-expressing breast cancers (1+/2+) either lacked nuclear Hdm2 expression (0) (49%, *n* = 35), as opposed to 51% (*n* = 37) expressing nuclear Hdm2 (1+/2+) (Table 3). Nuclear p14<sup>ARF</sup> showed no significant associations with nuclear Hdm2 expression (Fig. 2a). Surprisingly, the minority of cytoplasmic ARF-expressing breast cancers showed a significant association between increasing levels (MQS) of cytoplasmic p14<sup>ARF</sup> and nuclear Hdm2 expression (cc 0.475, *P* < 0.001) (Fig. 2c), as well as cytoplasmic Hdm2 expression (cc 0.461, *P* < 0.001) (Fig. 2d). Cytoplasmic ARF showed no other significant associations with p53. There were no associations between nuclear p53 and Hdm2 levels of expression or subcellular localisation (Table 3).

#### Association of p14<sup>ARF</sup>, p53 and Hdm2 with clinicopathological factors

We investigated the relationships between nuclear and cytoplasmic levels of p14<sup>ARF</sup> expression and clinicopathological parameters (listed in Table 1), with ARF MQSs analysed as a continuum for the purposes of statistical

analysis. There were no significant associations between either nuclear or cytoplasmic ARF with established prognostic indicators in invasive disease (lymph node involvement, large tumour size > 2 cm, increasing tumour histological grade, ER negativity, lymphovascular invasion, NPI and increased proliferation/Ki67), or in DCIS (Van Nuys Pathologic Classification, ER negativity and increased proliferation/Ki67). Nuclear ARF expression (defined as positive [MQS ≥ 3] or negative [MQS < 3]) showed a statistical relationship to HER-2 positivity (2+/3+) in IDCs ( $\chi^2$  test, *P* = 0.038); however, this relates to a limited number of HER-2-expressing breast cancers (*n* = 14) in association with ARF. A further subset analysis of levels of p14<sup>ARF</sup> expression (negative [0–2], versus weak/moderate [3–5] versus strong [6–8]; Table 2) in relation to clinicopathological parameters (data not shown) showed no significant associations.

Nuclear and cytoplasmic levels of p53 expression (assessed as a MQS continuum) were analysed in relation to clinicopathological criteria as for p14<sup>ARF</sup>. Nuclear p53 levels were significantly associated with increased proliferation (Ki67) (cc 0.275, *P* = 0.008) in IDCs, with cytoplasmic p53 showing a similar association in DCIS (cc 0.295, *P* = 0.005). There was a possible significant trend in IDCs between nuclear p53 and the NPI (cc 0.210, *P* = 0.063).

**Table 4****Relationships between clinicopathological variables, subcellular Hdm2 and patient outcome**

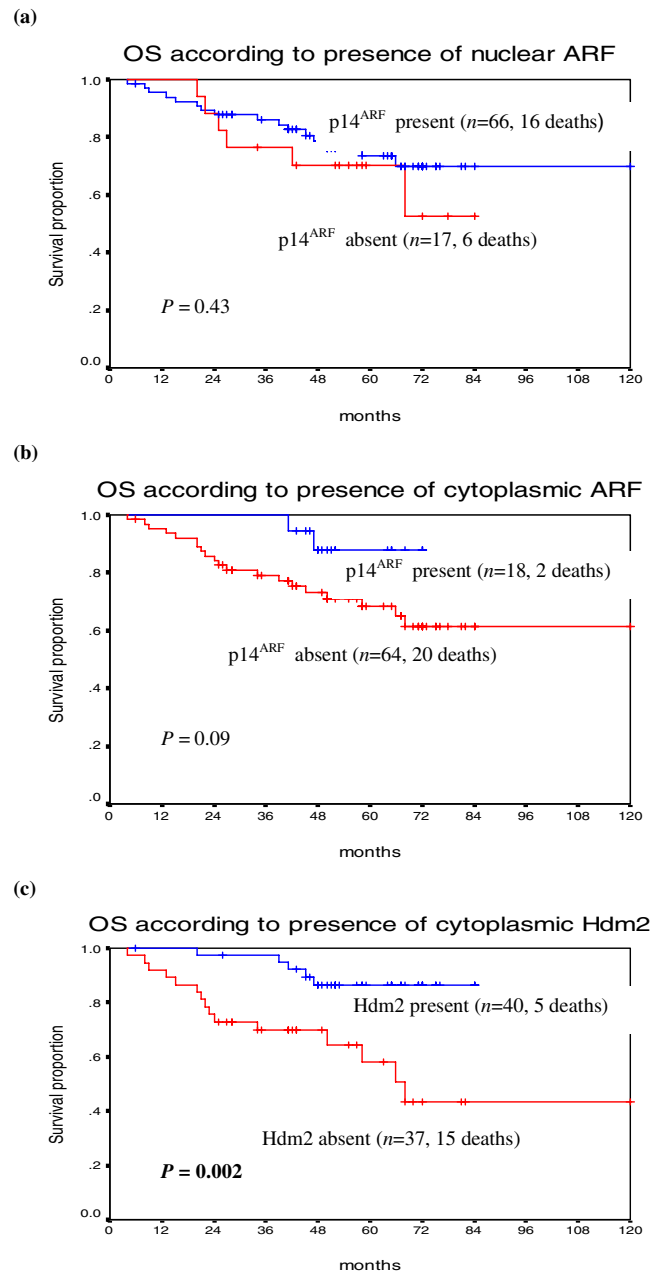
Variable	Univariate analysis		Multivariate analysis	
	95% CI	<i>P</i>	95% CI	<i>P</i>
Overall survival				
Age	0.99–1.04	0.4	0.98–2.90	<b>0.009</b>
Lymph node status (+/-)	2.79–153.9	<b>0.004</b>	2.65–161	<b>0.003</b>
Invasive tumour grade (I, II, III)	1.27–4.93	<b>0.002</b>	1.14–4.62	<b>0.002</b>
Tumour size (cm)	1.02–1.05	<b>&lt;0.001</b>	1.02–1.05	<b>&lt;0.001</b>
NPI	1.48–3.36	<b>&lt;0.001</b>	1.39–3.32	<b>&lt;0.001</b>
ER quick-score	0.66–0.92	<b>0.002</b>	0.68–0.98	<b>0.03</b>
HER-2 score	0.94–2.02	<b>0.04</b>	0.76–1.95	0.56
Ki67 proliferative index	0.99–1.00	0.4	0.99–1.00	0.65
Lymphovascular invasion (+/-)	1.46–9.38	<b>0.006</b>	0.89–6.69	0.08
Hdm2 cytoplasm MQS	0.66–0.95	<b>0.01</b>	0.67–0.97	<b>0.02</b>
Hdm2 nucleus MQS	0.68–1.01	0.07	0.69–1.05	0.13
Disease-free survival				
Age	0.97–1.02	0.9	0.99–1.06	0.06
Lymph node status (+/-)	2.29–25.3	<b>&lt;0.001</b>	2.06–25.6	<b>0.002</b>
Invasive tumour grade (I, II, III)	1.48–5.38	<b>0.005</b>	1.36–5.11	<b>0.006</b>
Tumour size (cm)	1.02–1.04	<b>&lt;0.001</b>	1.02–1.04	<b>&lt;0.001</b>
NPI	1.46–3.17	<b>&lt;0.001</b>	1.38–3.15	<b>&lt;0.001</b>
ER quick-score	0.66–0.89	<b>0.001</b>	0.68–0.95	<b>0.01</b>
HER-2 score	1.04–1.92	<b>0.02</b>	0.95–1.87	0.2
Ki67 Proliferative index	0.99–1.00	0.2	0.99–1.00	0.3
Lymphovascular invasion (+/-)	1.63–6.95	<b>0.002</b>	1.09–6.65	<b>0.03</b>
Hdm2 cytoplasm MQS	0.73–1.02	<b>0.05</b>	0.70–0.98	<b>0.03</b>
Hdm2 nucleus MQS	0.78–1.09	0.34	0.76–1.09	0.9

Confidence intervals (CI) and *P* values are given for the results of both the univariate and multivariate analyses. Data for the univariate analysis were evaluable in 97 patients (reflecting the exclusion of 6 local tumour recurrences as described in the Methods section) and included a multivariate analysis on 86 cases that excluded non-evaluable Nottingham Prognostic Index (NPI) (nodes, grade and size) in 11 patients. All clinicopathological variables and subcellular Hdm2 modified Quick-scores (MQSs) were analysed as a continuum, with lymph node status and lymphovascular invasion being assessed as present or absent. Univariate and multivariate analyses for cytoplasmic Hdm2 (and nuclear Hdm2) (overall survival) were evaluated on 77 and 70 patients, respectively (85 patients univariate [disease-free survival], and 77 patients multivariate, [disease-free survival]). The multivariate analysis is adjusted for NPI and treatment (tamoxifen/chemotherapy/none). Relationships that reached significance (*P* < 0.05) are highlighted in bold. ER, oestrogen receptor.

A subset analysis of p53 positivity (MQS  $\geq$  3) was evaluated in relation to clinicopathological parameters. This showed that nuclear p53 expression in invasive breast cancers (MQS  $\geq$  3 compared with MQS < 3) showed a significant correlation with loss of tumour differentiation (cc 0.243, *P* = 0.019).

We further analysed the relationship between nuclear and cytoplasmic Hdm2 expression and established parameters as described (Table 1), using the MQS as a continuum. In invasive tumours, increasing levels of nuclear and cytoplasmic

Hdm2 expression were significantly associated with increased proliferation (Ki67) (cc 0.281, *P* = 0.007, and cc 0.224, *P* = 0.032, respectively). In DCIS nuclear Hdm2 was associated with HER-2 overexpression (2+/3+) (cc 0.249, *P* = 0.019), with no similar association with cytoplasmic Hdm2 (cc 0.181, *P* = 0.09). A further evaluation of Hdm2 positivity (MQS  $\geq$  3) to described parameters showed no other significant associations.

**Figure 3**

Relationships between p14<sup>ARF</sup> and Hdm2 localisation and overall survival. Overall survival curves according to nuclear p14<sup>ARF</sup> (a), cytoplasmic p14<sup>ARF</sup> (b) and cytoplasmic Hdm2 (c), with patient numbers and deaths in parentheses. The classification of 'present' or 'absent' p14<sup>ARF</sup> or Hdm2 expression was determined by an MQS of 3 or more to define positivity, compared with an MQS of <3 to define absent expression. Patient numbers reflect the exclusion of six local tumour recurrences from the analysis of patient outcome and include only recorded deaths in 23 patients. *P* values are given for the log ranks that reached significance.

### Relationship of clinicopathological factors to prognosis and the predictive potential of p14<sup>ARF</sup> in relation to p53 and Hdm2 expression

Overall survival (OS) and disease-free survival (DFS) were determined in 89 and 97 patients, respectively (primary tumours), with a median follow-up of 51 months (range 4–120 months). Disease relapses (local or distant recurrences) occurred in 30 women; of these, death was confirmed in 23 patients, with 8 suspected deaths in the absence of a recorded mortality date. Locoregional recurrence occurred at a median duration of 28.5 months (range 3–120 months) from diagnosis. Breast cancer-related mortality occurred at a median of 26 months (range 8–98 months) from presentation. The mean duration of OS and DFS were 93 months and 87 months, respectively. Four-year DFS and OS were 70% and 77%, respectively. The relationship of established clinicopathological features to OS and DFS were analysed with Cox's regression analysis (Table 4). Generally poor prognostic factors such as large tumour size, high tumour grade, lymph node metastases, ER negativity, HER-2 overexpression and NPI were significantly associated with decreased OS and DFS. High tumour proliferation (Ki67 on immunohistochemistry), although associated with a smaller percentage of patients remaining disease-free (DFS) and alive (OS) at 4 years, did not reach statistical significance on univariate or multivariate analysis.

Univariate and multivariate analysis with the continuous MQS variables were used to investigate possible relationships between patient outcome data and levels of subcellular expression for p14<sup>ARF</sup>, Hdm2 and p53. When scores were measured as a continuum, neither nuclear nor cytoplasmic ARF expression was predictive of OS or DFS after univariate or multivariate analysis (data not shown). Similarly, a Kaplan–Meier analysis for OS and DFS of the nuclear p14<sup>ARF</sup> subgroups (negative [0–2], weak/moderate [3–5] and strong [6–8]) showed no significant relationship (data not shown). The small number of patients with strong cytoplasmic p14<sup>ARF</sup> precluded a similar analysis (see Table 2). Kaplan–Meier survival curves showed that the presence of cytoplasmic p14<sup>ARF</sup> (MQS  $\geq 3$ ) might be associated with a better OS compared with ARF negativity (MQS < 3) ( $P = 0.09$ , Fig. 3b), with a similar separation of DFS curves, although not statistically significant (data not shown). The presence or absence of nuclear p14<sup>ARF</sup> (MQS  $\geq 3$  compared with MQS < 3) was not significantly associated with patient outcome (Fig. 3a).

Subcellular levels of p53 expression were not shown to be independent prognosticators for OS and for DFS on univariate or multivariate analyses (data not shown). Kaplan–Meier analyses of nuclear p53 subgroups (negative [0–2], weak/moderate [3–5] and strong [6–8]) showed a significant association between strong nuclear p53 expression

and a reduced DFS ( $P = 0.05$ ; log rank), although patient numbers were small for this group (data not shown). Similarly, nuclear p53 expression (MQS  $\geq 3$  compared with MQS  $< 3$ ) was associated with a reduced OS and DFS, although this was not statistically significant (data not shown). Nuclear Hdm2 expression was not an independent prognosticator for OS and for DFS on univariate or multivariate analyses. Cytoplasmic Hdm2 expression (analysed as a continuous MQS) was associated with improved OS ( $P = 0.01$ , univariate analysis) and DFS ( $P = 0.05$ , univariate) (Table 4). Cytoplasmic Hdm2 independently predicted for improved OS ( $P = 0.02$ , multivariate) and DFS ( $P = 0.03$ , multivariate) (Table 4). Kaplan–Meier survival curves showed that the presence of cytoplasmic Hdm2 (MQS  $\geq 3$  compared with MQS  $< 3$ ) was associated with improved OS ( $P = 0.002$ ) (Fig. 3c).

## Discussion

p14<sup>ARF</sup> suppresses cancer cell growth, and several *in vitro* studies have reported its inhibition of Hdm2 to stabilise and activate p53, with a loss of ARF increasing p53 degradation. The relevance of these mechanisms *in vivo* requires further investigation, in view of ARF's proposed function as a tumour suppressor and the suggestion that p14<sup>ARF</sup> overexpression is a surrogate marker of dysregulation of the pRb and p53 pathways [30]. p14<sup>ARF</sup> mRNA expression in breast cancers underlines a variability with overexpression in 17–19% and underexpression in 24–26% [38,39]. There are few studies clarifying p14<sup>ARF</sup> protein levels *in vivo*, with low ARF expression in non-neoplastic epithelium [17,34,35]. The present study suggests an increased frequency of nuclear p14<sup>ARF</sup> overexpression in 47% of invasive and non-invasive breast cancers. Nuclear p14<sup>ARF</sup> overexpression has been shown by immunohistochemistry in 22% of B cell lymphomas to predict tumour aggression and outcome [30]. Possible abnormalities in the p53 pathways might be implicated [10–14,30,34]. p53-independent mechanisms might also contribute through oncogenic stimulation of ARF [16,17,30].

The lack of p14<sup>ARF</sup> expression in 21% of tumours is not dissimilar to other studies of ARF mRNA levels in breast cancers [38,39]. B cell lymphomas lacked nuclear p14<sup>ARF</sup> in 11% of cases, in association in large part with promoter hypermethylation, as reported for breast cancers [21,30,38]. Predominant nucleolar localisation of p14<sup>ARF</sup> is determined by amino-terminus and exon 2 carboxy-terminus, with evidence of this after immunofluorescence of a number of cancer cell lines [25,26] and exclusive nucleolar p14<sup>ARF</sup> in a subset of lymphomas and non-tumour tissue [25,26,30]. The 4C6 monoclonal antibody and other p14<sup>ARF</sup> monoclonal antibodies have verified the nuclear/nucleoplasmic localisation of ARF with intactness of its functional pathway, as well as its implications as a prognostic surrogate marker compared with nucleolar ARF [27,30].

This study shows nuclear p14<sup>ARF</sup> in the majority of breast cancers, with 24% cytoplasmic detection. Few other studies have attempted to analyse cytoplasmic p14<sup>ARF</sup>, although detectable on immunohistochemistry, with *in vitro* evidence of an ARF-binding cytoplasmic protein [33–37]. Cytoplasmic p14<sup>ARF</sup> has been observed in non-small cell lung cancers, in oral squamous carcinomas and in another study of lung and pancreatic tumours with the use of several monoclonal and polyclonal antibodies [34–37]. This study analysed cytoplasmic ARF in the context of positive and sero-matched negative controls as described, with no evidence of ARF expression in surrounding normal breast epithelial cells. Such observations might invoke an additional pathway of ARF regulation through changes in its subcellular localisation as for other tumour suppressors, such as p53 and p21<sup>WAF1/CIP1</sup> [5,8,51,52]. Similar genomic aberrations might occur in DCIS and IDC, highlighting possible similarities in protein expression [53,54]. This study shows a consistent similarity in levels of protein expression and subcellular localisation for p14<sup>ARF</sup>, p53 and Hdm2 in DCIS and IDCs.

It is suggested that the upregulation of nuclear p14<sup>ARF</sup> expression is a consequence of cell cycle malfunction involving p53 and Hdm2 [30]. Increasing levels of nuclear ARF on immunohistochemistry are a measure of p53 inactivation by mutation, or by Hdm2 overexpression resulting in disruption of the p53–p14<sup>ARF</sup> negative feedback loop [30]. The implication is that p14<sup>ARF</sup> expression is associated with P53-deficient cell lines, suggesting a p53-mediated downregulation of ARF [10–14]. Although p53 immunostaining is not necessarily tightly correlated with TP53 gene function, 57% (17 cases) of p53-positive tumours in this study strongly expressed ARF, reflecting similar *in vivo* evidence showing concomitant p14<sup>ARF</sup> mRNA expression and p53 immunostaining in breast cancers, with a similar relationship between increasing levels of ARF protein and p53 mutations in B cell lymphomas (Table 3) [30,34,39]. Others have shown a lack of an inverse relationship between the two genes, suggesting that the ARF–p53 pathways are not strictly linear and that decreased ARF expression and TP53 mutations are not mutually exclusive [39,55]. ARF stabilises nuclear p53 and is postulated to congregate with Hdm2 and p53 in ternary complexes or 'nuclear bodies' that have yet to be demonstrated *in vivo* [10,25,27,30].

Double-fluorescent immunolabelling in B cell lymphomas revealed a partial co-localisation of nuclear p14<sup>ARF</sup> and p53, with no Hdm2 association, suggesting that the mechanisms underpinning ARF's role in inhibiting the nuclear export of p53 by Hdm2 inhibition remains to be defined [30]. Studies *in vivo* have not previously examined a possible association between ARF expression and p53 subcellular localisation in breast cancer. In large part, this

interpretation depends on the accumulation of p53 that is likely to reflect mutant p53 as inferred from immunohistochemistry [30,34,49]. Proportionately more (55%) p53-expressing breast cancers showed exclusive nuclear p53 in association with nuclear ARF expression (data not shown).

Hdm2 expression is a feature of tumorigenesis, with overexpression attributed in part to gene amplifications in the minority of breast cancers [56-59]. Other contributing mechanisms include enhanced transcription and translation and an extended protein half-life. This study demonstrates a minority (11%) of Hdm2-overexpressing (MOS 6-8) invasive breast cancers and 16% of non-invasive breast cancers. Recent evidence shows that p14<sup>ARF</sup> overexpression is associated with increased levels of Hdm2 in cancer cells, with a similar finding in oral cancers and B cell lymphomas that indicate a direct association between p14<sup>ARF</sup> and Hdm2 expression determined by immunohistochemistry [27,30,35]. Similar findings are reflected in this series of breast cancers (Table 3).

An additional mechanism of Hdm2-mediated regulation by p14<sup>ARF</sup> involves intracellular compartmentation [21]. The subcellular localisation of Hdm2 involves its nucleolar localisation signal, as well as both nuclear import and export receptors [59]. *In vitro* immunofluorescent studies verify the ARF-mediated localisation of Hdm2 in the nucleolus and nucleoplasm [27]. Not uncommonly in colorectal cell lines and primary tumours, *in vivo* inactivation of ARF by methylation is associated with increasing cytoplasmic Hdm2 expression, emphasising the importance of p14<sup>ARF</sup> in nuclear Hdm2 localisation [21]. ARF-negative cancers, implying inactivation in this series, show no specific association with increased cytoplasmic Hdm2 expression (Fig. 2). There is a further suggestion in this series of breast cancers that nuclear ARF expression is associated with both nuclear and cytoplasmic Hdm2-expressing breast cancers, underlining the absence of a preferential nuclear localisation of Hdm2 (data not shown). Similarly, a study of colorectal cancers shows the lack of an exclusive relationship between ARF function and the subcellular localisation of Hdm2, in comparison with cell lines [21]. HER-2/*neuneu* might also regulate the subcellular localisation of Hdm2, with better prognostic HER-2-negative breast cancers associated with Hdm2 in both the nucleus and cytoplasm, in contrast to HER-2-mediated phosphorylation of Hdm2 to produce its nuclear localisation and the degradation of p53 [60]. Such findings could imply that the relationship between p14<sup>ARF</sup> and the subcellular localisation of Hdm2 is not as exclusive as that found in cell lines.

Clinically, the implications of p14<sup>ARF</sup> levels of expression in breast cancer are unknown; two previous studies examined p14<sup>ARF</sup> mRNA expression in relation to prognostic

parameters [38,39]. Variable p14<sup>ARF</sup> mRNA expression including both overexpression as well as decreased mRNA levels are reported to be significantly associated with poor prognostic criteria including p53 mutational status, peritumoural vessel invasion, lymph node metastases and negative progesterone receptors [38,39]. By comparison, nuclear p14<sup>ARF</sup> overexpression in B cell lymphomas with the use of immunohistochemistry predicts tumour aggression and reduced overall survival [30]. We find no clear associations between ARF levels and prognostic parameters or outcome, although the presence of cytoplasmic p14<sup>ARF</sup> as opposed to its absence suggests a better outcome (Fig. 3b). At present, a poor understanding of the implications of cytoplasmic ARF expression in tumours requires further study [35-37]. *In vitro*, HER-2/*neuneu* interacts with the p14<sup>ARF</sup>/Hdm2 pathway; some *in vivo* studies show that p14<sup>ARF</sup> mRNA overexpression in breast cancers is correlated with HER-2 negativity [38,39,61]. *In vitro*, HER-2 promotes Hdm2-mediated p53 degradation through the inactivation of ARF, and HER-2 further enhances mammary tumorigenesis in ARF heterozygous mice [60,61]. Our finding of an association between ARF and HER-2 expression is preliminary and suggests the importance of HER-2 in the ARF pathway.

Inactivation of p53 might occur through pathways other than mutation, involving p14<sup>ARF</sup>/Hdm2 and its degradation through subcellular localisation. Immunohistochemical p53 expression might detect up to 89% of TP53 point mutations in breast carcinoma specimens, although it does not always correlate with specific mutations in exons 5 to 9 [34,49]. Despite an established body of literature regarding the implications of p53 nuclear immunoreactivity, few or no *in vivo* studies have evaluated cytoplasmic p53. The cytoplasmic localisation of p53 is a prerequisite for its proteosomal degradation and has been implicated in patient prognosis [5,62]. A study of inflammatory breast cancers showed the presence of wild-type cytoplasmic p53 in 37% of cases [5]. We found a significant association of cytoplasmic p53 with increased tumour proliferation in DCIS, suggesting the value of its further investigation in future studies. Similarly, Hdm2 undergoes nucleocytoplasmic shuttling; highly proliferative, invasive breast cancers were associated with increasing levels of nuclear or cytoplasmic Hdm2 expression in this study. Immunohistochemical p53 and Hdm2 expression were also shown to be correlated with ki67 in B cell lymphomas [30]. A possible functional relevance of subcellular Hdm2 expression is suggested, although Ki67 was not an independent prognosticator in this study.

Our findings of an association between HER-2 and nuclear Hdm2 expression might support *in vivo* findings in which HER-2, through phosphoinositide 3-kinase/Akt-mediated phosphorylation, is preferentially associated with nuclear

Hdm2 [60]. In the present study the association between the presence of cytoplasmic Hdm2 and improved outcome would initially seem counterintuitive, yet it is substantiated by recent evidence that HER-2-negative and Akt-negative cell lines are associated predominantly with cytoplasmic Hdm2 [60]. *In vivo*, the study of a small subset of breast cancers (21 tumours) confirmed these *in vitro* findings, suggesting an important relationship between HER-2 activation and the p14<sup>ARF</sup>/Hdm2 and p53 pathways [60].

## Conclusion

Defining relevant pathways regulating p53 function are fundamental to an understanding of its tumour suppressor role and mechanisms of p53 inactivation, which might be independent of a p53 mutation. p14<sup>ARF</sup> and Hdm2 are key upstream and downstream regulators of p53, although in part they might be independent of p53. We have demonstrated a spectrum of tumour-specific p14<sup>ARF</sup> upregulation with predominant nuclear ARF expression in the absence of exclusive nucleolar staining in invasive and non-invasive breast cancers; we suggest that the presence of cytoplasmic p14<sup>ARF</sup> might be favourable in terms of breast cancer outcome. Predominant nuclear ARF levels showed no clear clinical implications about the degree of ARF expression versus its negativity. Clearly our *in vivo* findings do not completely mirror *in vitro* relationships between p14<sup>ARF</sup>/Hdm2 and p53 to suggest a greater complexity of these pathways in human solid tumours. Increasingly, evidence suggests the importance of evaluating the subcellular localisation of checkpoint proteins to include p53, Hdm2 and p14<sup>ARF</sup> [51,52]. Potentially, changes in the cellular distribution of these proteins might be driven by key tumorigenic processes to which HER-2 and other growth factor pathways might contribute.

## Competing interests

None declared.

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