

P14^{ARF} Is Down-regulated During Tumour Progression and Predicts the Clinical Outcome in Human Breast Cancer

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Abstract. The objective of this study was to determine the mRNA expression for p14 and p16 in a cohort of women with breast cancer. *Materials and Methods:* Breast cancer specimens (N= 127) and normal tissue (N=23) specimens were studied. Transcript levels were determined using quantitative polymerase chain reaction (PCR), and were correlated with clinicopathological data collected over 10 years. *Results:* Higher p14 mRNA transcript levels were associated with non-cancerous background tissue specimens (median copy numbers: 103 vs. 4, $p=0.0095$), with better overall and disease-free survival, and in TNM2 stage tumours (TNM2 vs. TNM1, 27.2 vs. 3.5, $p=0.049$; TNM1/TNM2 vs. TNM3/4, 26 vs. 2, $p=0.009$). There was no significant relationship between p16 levels and clinicopathological parameters. A correlation between p14 and human telomerase reverse transcriptase (hTERT) levels was observed ($r=0.406$, $p=0.00005$). *Conclusion:* p14 expression seems to increase initially in early breast cancer and decrease with further tumour progression. p14 may be induced to counteract immortalisation and hTERT surge.

The cyclin-dependent kinase-2A (CDKN2A, or INK4A)/alternative reading frame (ARF) locus at chromosome 9p21 is crucial in the pathogenesis of several types of malignant disorders and encodes two unrelated cell-cycle regulators, p14^{ARF} and p16^{INK4A}, which have been regarded as significant senescence markers (1).

The objective of this study was to determine the mRNA expression for p14 and p16 in a cohort of women with breast

cancer. The expression levels were compared with normal background tissues and evaluated against established pathological parameters and the clinical outcome over a median follow-up period of 120 months.

Materials and Methods

Samples. Tissue samples were collected after informed consent with ethical approval as per contemporaneous institutional guidelines. Immediately after surgical excision, a tumour sample was obtained from the tumour area, while another was obtained from the associated non-cancerous tissue (ANCT) within 2 cm of the tumour, without affecting the assessment of tumour margins. Breast cancer tissues (n=127) and normal background tissues (n=23) were collected and stored at -80°C in liquid nitrogen until the commencement of this study. This cohort has been the subject of several completed and current studies (2, 3).

All the patients were treated according to local guidelines, following discussions in multidisciplinary meetings. Patients undergoing breast-conserving surgery also underwent radiotherapy. Hormone-sensitive patients were given tamoxifen. Hormone-insensitive cases, high-grade cancer, and node-positive cases were treated with adjuvant therapy. Clinicopathological data (Table I) was collected from the patient charts, and was collated in an encrypted database. The median follow-up period was 120 months (June 2004).

RNA extraction kits and reverse transcription kits were obtained from AbGene Ltd. (Epsom, Surrey, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Invitrogen Ltd. (Paisley, United Kingdom). Custom made hot-start Master Mix for quantitative PCR was from AbGene (4).

Tissue processing, RNA extraction and cDNA synthesis. Approximately 10 mg of cancerous tissue were homogenised. A larger amount of ANCT (20-50 mg) was used as its high fat content made it difficult to obtain sufficient RNA for analysis. The concentration of RNA was determined using a UV spectrophotometer (Wolf Laboratories, York, UK) to ensure adequate amounts of RNA for analysis. Reverse transcription was carried out using a reverse transcription kit (AbGene) with an

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Table I. Clinical data describing the patient cohort.

Parameter	Category	Number
Node status	Node positive	48
	Node negative	76
Tumour grade	1	14
	2	36
	3	46
	4	4
Tumour type	Ductal	74
	Lobular	12
	Medullary	2
	Tubular	1
	Mucinous	2
	Other	5
TNM staging	1	50
	2	34
	3	5
	4	4
Clinical outcome	Disease-free	69
	With local recurrence	5
	Alive with metastasis	4
	Died of breast cancer	13
Follow-up	Median (in months)	120
	Range (in months)	110 to 150

anchored oligo (dT) primer, using 1 mg of total RNA in a 96-well plate to produce cDNA. The quality of cDNA was verified using β -actin primers (primers 5'-ATGATATCGCCGCTCGTC-3' and 5'-CGCTCGGTGAGGATCTTCA-3') (4).

Quantitative analysis. Transcripts of cDNA library were determined using real-time quantitative polymerase chain reaction (qPCR) based on Amplifluor technology. The PCR primers were designed using Beacon Designer software (Premier Biosoft International Ltd., Pal Alto, CA, USA), but an additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACA-3'), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added to the primer. The primers used are detailed in Table II. The reaction was carried out under the following conditions: 94°C for 12 min and 50 cycles of 94°C for 15 s, 55°C for 40 s, and 72°C for 20 s. The levels of each transcript were generated from a standard that was simultaneously amplified within the samples. Levels of transcripts were normalised against cytokeratin 19 (CK19) With every run of the PCR, a negative and positive control was employed, using a known cDNA sequence (podoplanin) (4).

Statistical analysis. Analysis of the data was performed using the Minitab 12 statistical software package (Minitab Ltd., Coventry, UK.) using a custom-written macro (Stat06e.mtb). Medians were compared using the Mann-Whitney *U*-test, while means were compared using the two-sample *t*-test. The transcript levels within the breast cancer specimens were compared to those of the ANCT and correlated with clinicopathological data collected over a 10-year follow-up period.

p-Values less than 0.05 were considered significant, whereas *p*-values between 0.05 and 0.10 were considered marginally significant.

Table II. Primers used in the study.

Gene	Sequence (5'-3')
<i>P14</i> Forward	tttctgtgttcacatcc
<i>P14</i> Z Reverse	actgaacctgaccgtacaggctgctgcctagac
<i>GAPDH</i> Forward	ctgagtacgtcgtggagtc
<i>GAPDH</i> Z Reverse	actgaacctgaccgtacacagatgatgaccttttg
<i>CK19</i> Forward	caggtccgaggttactgac
<i>CK19</i> Z Reverse	actgaacctgaccgtacacactttctgccagtgtgtcttc
<i>hTERT</i> Forward	gtggatgattcttctgttgg
<i>hTERT</i> Z Reverse	actgaacctgaccgtacaagggtgagactggctctgat
β -actin, Forward	atgatatcgccgcgctcgtc
β -actin, Reverse	cgctcgttgaggatcttca

Table III. Comparison of *p14ARF* mRNA expression levels (median copy numbers normalised against cytokeratin 19) in subgroups within cohort.

Patient and tumour characteristic	Median values (normalised copy numbers)	95% Confidence interval (normalised copy numbers)	<i>p</i> -Value
Tumour grade			
1 vs. 2	19 vs. 3	-2.7 vs. 34.3	0.2658
1 vs. 3	19 vs. 3.9	-6.1 vs. 33.8	0.5010
2 vs. 3	3 vs. 3.9	-5.4 vs. 1.8	0.6237
NPI			
1 vs. 2	9.6 vs. 3	-0.4 vs. 14.6	0.1921
1 vs. 3	9.6 vs. 0.3	-0.0 vs. 33.7	0.0649
2 vs. 3	3 vs. 0.3	-0.4 vs. 6.2	0.3415
TNM			
1 vs. 2	3.5 vs. 27.2	-43.4 vs. -0.1	0.0490
1 vs. 3	3.5 vs. 0.004	-0.0 vs. 114.4	0.0350
1 vs. 4	3.5 vs. 0.3	-0.4 vs. 190.0	0.3299
2 vs. 3	27.2 vs. 0.004	0.3 vs. 789.7	0.0037
2 vs. 4	27.2 vs. 0.3	-0.1 vs. 811.2	0.0457
3 vs. 4	0.004 vs. 0.3	-9.547 vs. 1.028	0.5403
Survival			
DF vs. LR	4.7 vs. 1.7	-98.6 vs. 33.6	0.9656
DF vs. DR	4.7 vs. 395.3	-1362.6 vs. 61.9	0.5855
DF vs. D	4.7 vs. 0.8	-0.3 vs. 14.0	0.2403
DF vs. LR/DR/D	4.7 vs. 1.3	-0.8 vs. 7.7	0.5346

DF: Disease-free survival, LR: local disease recurrence, DR: distant disease recurrence, D: death from breast cancer, NPI: Nottingham prognostic index, TNM: TNM: Clinical stage according to Tumour size, Nodal status and presence of distant Metastases.

Table IV. Spearman rank-order correlations.

		<i>p14</i>	<i>p16</i>	<i>hTERT</i>
<i>p12</i>	Correlation coefficient	0.174	0.123	0.0556
	<i>p</i> -Value	0.0891	0.229	0.594
<i>p14</i>	Correlation coefficient		0.0655	0.406
	<i>p</i> -Value		0.523	0.0000549
<i>p16</i>	Correlation coefficient			-0.0330
	<i>p</i> -Value			0.751

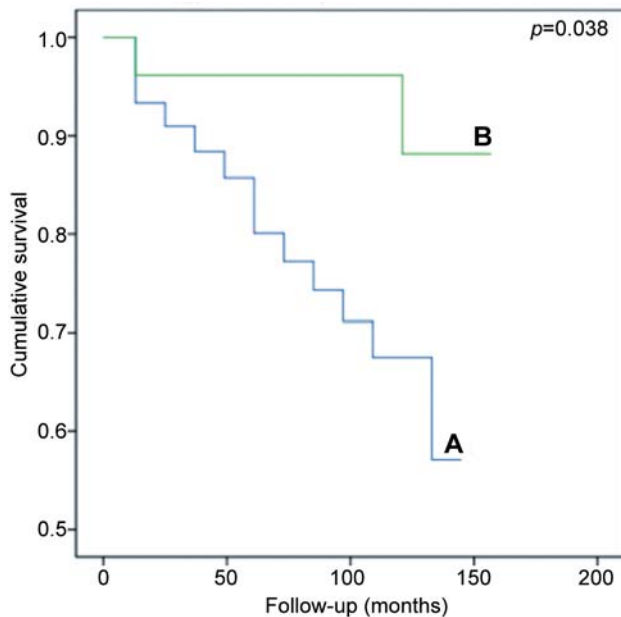


Figure 1. Disease-free survival curve according to mRNA expression of *GNB1*. The population has been divided into higher and lower transcription groups with the moderate prognosis group by Nottingham prognosis index (NPI) serving as the dividing line. Curve A: Lower transcription group; Curve B: higher transcription group.

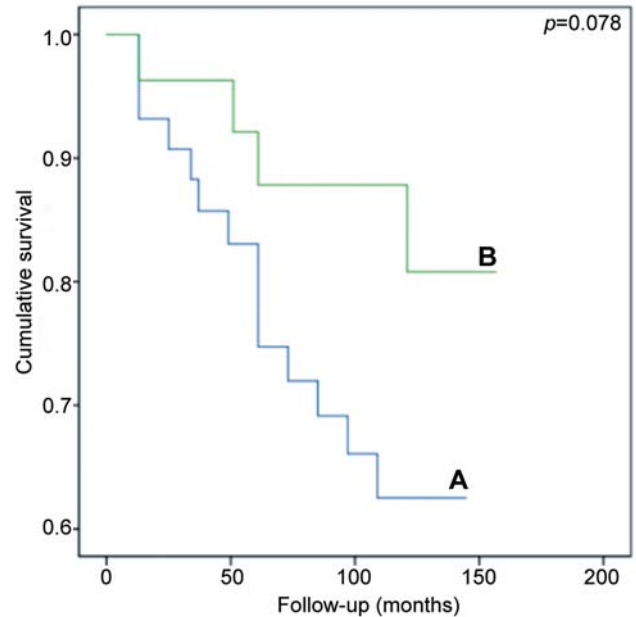


Figure 2. Overall survival curve according to mRNA expression of *GNB1*. The population was divided into higher and lower transcription groups with the moderate prognosis group by Nottingham prognosis index (NPI) serving as the dividing line. Curve A: Lower transcription group; Curve B: higher transcription group.

Correlations between the expressions of the molecules were studied using Spearman rank-order correlation test.

For purposes of the Kaplan–Meier survival analysis, the samples were divided arbitrarily into high and low transcription groups, with the value for the moderate prognostic group as defined by NPI serving as the dividing line. Survival analyses were performed using PSAW18 (SPSS Inc., Chicago, IL, USA).

Results

Higher mRNA transcript levels were found for *p14* in the background tissue compared to the breast cancer specimens (median values: 103 vs. 4, $p=0.0095$). The *p14* expression level was significantly higher in TNM2-stage tumours compared with TNM1 tumours (27.2 vs. 3.5, $p=0.049$). The levels decreased significantly with further tumour progression through TNM3 and TNM4 (TNM1/TNM2 vs. TNM3/4, 26 vs. 2, $p=0.009$) (Table III).

After a median follow-up of 10-years, higher *p14* expression levels were significantly associated with better overall survival and disease-free survival (Figures 1 and 2).

There was no significant relationship between *p16* levels and clinicopathological parameters or clinical outcome.

Furthermore, we observed a highly significant positive correlation between *p14* and hTERT levels, both normalised

against the epithelial marker CK19 ($r=0.406$, $p=0.00005$). There was a weak negative and non-significant negative correlation between *p16* and hTERT ($r=-0.033$, $p=0.71$). (Table IV)

Discussion

The *INK4A/ARF/INK4B* locus at chromosome 9p21 encodes for three proteins with a role in the control of the cell cycle. Two of these, *p16* and *p15*, are products of the *INK4A* and *INK4B* regions of this locus, which are separated by an intervening exon termed *1β*. Both of these proteins have a role in the control of the retinoblastoma (Rb) family of cycle regulators (*pRb*, *p107* and *p130*) (5).

Under normal conditions, the Rb proteins inhibit DNA synthesis. In the course of the cell cycle, this action is inhibited when Rb proteins are phosphorylated by cyclin dependent kinase (CDK) -4 and -6, thereby facilitating progression of the cell from the first gap (G_1) phase to the synthetic (S) phase of the cell cycle. *p16* and *p15* inhibit this action of CDK4/6 on Rb, thus enabling exit from the cell cycle (5, 6).

This Rb pathway is known to be downstream of the rat sarcoma (RAS) cascade, and the phosphatidylinositol 3-kinase (PI3K) signalling pathway. These pathways have well-

known roles in a number of neoplastic diseases (7, 8). p16 has been found to have a role in a number of malignancies, including uveal melanoma, head and neck neoplasia, mesothelioma, and acute lymphoid leukaemia (9-13).

In the context of breast cancer, p16 is of interest as a member of the wider Rb pathway, which is known to have a role in invasive breast cancer, as well as in ductal carcinoma *in situ* (DCIS) (14). In a study of public microarray databases, Ertel *et al.* determined that Rb-related pathway related disruption was seen more commonly in cancerous tissue. In sub-group analysis, it was further determined that oestrogen receptor (ER)-negative tumours were more likely to have elevated p16 expression in conjunction with pRb loss, possibly reflecting a defect in Rb-mediated transcription despite elevated p16 levels. The role of the Rb pathway in oncogenesis has led to interest in p16 and other members of the pathway as markers for prognosis (15).

Witkiewicz *et al.* found elevated expression of p16 to be associated with Rb pathway disruptions in the context of DCIS. In addition, they confirmed an association between increased levels of p16 and ER-negative lesions. High p16 expression in conjunction with high Ki76 expression, as determined by histological staining, was found to be significantly predictive for the recurrence of DCIS (6).

However, in a study of breast tissues with atypical hyperplasia, a precursor to carcinoma *in situ*, Radisky *et al.* were unable to find an independent association between immunohistochemical staining for p16 and long-term disease prognosis and progression (16). Our findings corroborate this with regards to the mRNA expression of p16, as determined by quantitative PCR.

p16 has been characterized as a marker of cell senescence. Several studies have shown an association between p16 expression and patient age (16). p16 has been studied for its role in cell senescence. Cells can undergo replicative senescence mediated by the erosion of telomeres after a number of cell cycles, or be subject to senescence induced by cellular stress in a telomere-independent manner. The latter is termed as stress-induced premature senescence (SIPS), and is believed to be mediated by p16 (17). Studies in human lung fibroblasts have demonstrated that p16-related senescence is unaffected by the actions of hTERT (17, 18). In addition, suppression of p16 did not prevent replicative senescence in human fibroblasts (17, 19). Our findings are in keeping with these observations, with the weak correlation between p16 and hTERT likely to be due to upstream convergence in the PI3K pathway (20).

Transcription of the INK4A/ARF by a specific promoter invokes an *alternative reading frame* resulting in an additional protein termed p14, or ARF. p14 has a role in cell-cycle regulation, cell senescence, and apoptosis which is distinct from that of p16. It is characterized as a 15-kDa nucleolar protein, believed to exert the majority of its function through

the regulation of p53, which has a key role in DNA repair, cell-cycle regulation and metabolism (21).

The actions of p53 are suppressed by mouse double minute-2 (MDM2, also known as HDM2), which facilitates the translocation and eventual destruction of p53 in the cytosol. In turn, the transcription of MDM2 is controlled by p53, thereby completing a negative feedback loop. p14 inhibits the effect of MDM2 on p53 by sequestering MDM2 in the nucleolus (22). In addition, p14 inhibits the action of ARF-BP1, a E3 ubiquitin ligase which targets p53 (23).

Disruptions in the p53 pathway are widely observed in human oncogenesis, including breast cancer. The p53 pathway disruptions due deletion or silencing of p14 have been found in a number of neoplasias including those of the lungs, head and neck, colon, liver, and breast (21). Vestey *et al.* found a weak association between cytosolic p14 protein expressions in human breast cancer and overall survival (24). Aberrant methylation of p14 and p16 were shown to be associated with worse prognosis in colonic, breast and bladder carcinomas (25). Our findings regarding the prognostic implications of p14 expression are broadly in keeping with previous studies. However, our study is the first in recent literature to suggest a surge in p14 expression in early-stage human breast cancer.

In addition, p14 may form a part of a cell senescence pathway *via* p53, which stimulates pRb through p21. In contrast to the p16-Rb pathway, the p53-p21-pRb pathway mediates replicative senescence rather than SIPS (26). Shamanin *et al.* found that breast cell lines immortalised by hTERT underexpressed p14, and underwent p53-induced senescence if p14 expression was enforced (27).

Our results suggest a strong-positive correlation of p14 with hTERT. Taken together with the contrasting prognostic implications of hTERT and p14 (28), it may be surmised that p14 may be induced in order to offset the oncogenic effects of immortalisation due to hTERT.

Conclusion

p14 expression seems to increase initially with tumour progression in early breast cancer and subsequently seems to decrease with further tumour progression in advanced tumours. Tumours that maintain a high expression of p14 are associated with a superior clinical outcome.

p14 may be induced in early breast cancer in order to counteract immortalisation and hTERT surge. We believe that this is a novel finding in human breast cancer, meriting further investigation in order to better-understand the role of cell senescence pathways in human breast cancer pathogenesis.

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