mRNA Expression of *CDK2AP1* in Human Breast Cancer: Correlation with Clinical and Pathological Parameters

RITIKA GERA¹, LEON MOKBEL¹, WEN G. JIANG² and KEFAH MOKBEL¹

¹The London Breast Institute, The Princess Grace Hospital, London, U.K.;

²Metastasis & Angiogenesis Research Group, University Department of Surgery, Cardiff University School of Medicine, Cardiff University, Cardiff, U.K.

Abstract. *Background:* Cyclin-dependent kinase 2-associated protein 1 (*CDK2AP1*) interacts with *CDK2AP2*, modulates the actions of transforming growth factor- β 1, cyclin-dependent kinase 2 and retinoblastoma protein, and closely interacts with micro-RNA21 and micro-RNA25. Our objective was to determine if *CDK2AP1* mRNA expression levels were consistent with tumour-suppressive functions in breast cancer. *Materials and Methods:* A total of 134 samples were analysed. *CDK2AP1* mRNA levels were measured using quantitative polymerase chain reaction (RT-PCR) and normalised against glyceraldehyde 3-phosphate dehydrogenase mRNA. Levels in breast cancer and adjacent non-cancerous breast tissue were analysed against pathological and clinical parameters (TNM staging, survival over a 10-year follow-up period). *Results:* Normalised *CDK2AP1* expression was 38-fold higher in adjacent non-cancerous breast tissue than in breast cancer. *CDK2AP1* expression in disease-free patients at 10 years was more than threefold that of patients who died of breast cancer. However, neither of these differences in expression levels reached statistical significance. *CDK2AP1* mRNA levels were higher in TNM1 compared to TNM3 ($p=0.016$) and with TNM4 ($p=0.016$). There were no significant associations between *CDK2AP1* expression and estrogen receptor status, tumour grade and tumour type. There was no significant difference in overall survival between patients with high and those with

low *CDK2AP1* mRNA levels after a median follow-up of 10 years (Kaplan–Meier analysis, $p=0.872$). *Conclusion:* To our knowledge, this is the first study in the literature to examine the mRNA expression of *CDK2AP1* in human breast cancer over a long-term follow-up period. A compelling relationship exists between high *CDK2AP1* mRNA expression and lower TNM classification of breast cancer, which is consistent with *CDK2AP1* having a tumour-suppressive function.

Cyclin-dependent kinase 2-associated protein 1 (*CDK2AP1*) is hypothesised to be a tumour suppressor which works through the mediation of several other genes and proteins. First discovered in 1995 as ‘deleted in oral cancer-1’ (*DOC1*) gene (1), investigators have since found evidence for its growth-/tumour-suppressive action and confirmed it to be likely implicated in the development of many cancer types (2, 3).

CDK2AP1 and its associated homolog *CDK2AP2* likely work together to effectively suppress cell growth due to their probable structural and functional relation (4); *CDK2AP2* is a known tumour suppressor (5) which we previously investigated and provided evidence for its tumour-suppressive function in human breast cancer. The interaction between the two was recorded by using *CDK2AP1* as ‘bait’ in liver cDNA screening and was verified both *in vitro* and in cells (6).

Multiple investigations have been conducted regarding the relationship between different micro-RNAs (miRs) and *CDK2AP1*. Using cells taken from the tumour-free surgical margins of 18 patients with head and neck squamous cell carcinomas, *miR-21* was found to be inversely correlated with *CDK2AP1* and stimulate cell proliferation and invasion (7). Similarly, *miR-205* was found to suppress *CDK2AP1* and promote the invasion and proliferation of laryngeal squamous cell carcinoma (8). This suggests that *CDK2AP1* expression is intricately linked to miR expression and the inverse relationship found to exist between the two underlines its likely function of *CDK2AP1* as a tumour suppressor.

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Correspondence to: Kefah Mokbel, The London Breast Institute, The Princess Grace Hospital, 42-52 Nottingham Place, London W1U5NY, U.K. Tel: +44 2079082040, Fax: +44 2079082275, e-mail: kefahmokbel@hotmail.com

Key Words: Breast cancer, human breast cancer, tumour-suppressive function, tumour suppressor role, breast cancer specimen, *CDK2AP1*.

Table I. Primers used in quantitative real-time polymerase chain reaction.

Gene symbol	Gene name	Primer
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	actgaacctgaccgtacacagatgatgaccttttg
<i>CDK2AP1</i>	Cyclin-dependent kinase 2-associated protein 1	F1: aggtagcgcttcgattct Zr1: actgaacctgaccgtacatcctcttccttggtttcc F2: tctggaggacgaagtttg Zr2: actgaacctgaccgtacacaggaggaggtctgtgatt

CDK2AP1 is likely to work, at least in part, by mediating transforming growth factor- β 1 (TGF- β 1) to act as a growth suppressor, which in turn permits the modulation of CDK2AP1 and retinoblastoma protein. A study by Hu *et al.* was conducted by genetically engineering mouse oral keratinocytes which were CDK2AP1-deficient. It was found that CDK2AP1-deficient keratinocytes had twice the number of cells in S-phase, but fewer in the G₂ phase compared to wild-type cells (9). This suggested that TGF- β 1-mediated growth suppression was compromised, with reduced sensitivity to TGF- β 1 inhibition and increased CDK2AP1 activity.

There are many consequences of mutations within *CDK2AP1*. CDK2AP1 is normally able to dimerise to the homodimeric p25. However, if the dimerization is abolished, for example by inducing the C105A mutation, the mutated CDK2AP1 has a reduced growth-and CDK2-inhibitory effect (10). This suggests that mutated CDK2AP1 which lacks dimerization could potentially predispose a patient to cancerous growth due to a lack of growth suppression. Deletion mutations in the poly (T) 8 repeat sequence within the 3' of *CDK2AP1* results in functionally significantly reduced expression in human microsatellite-unstable colorectal cancer (11). This is caused, at least in part, by reduced mRNA stability.

The above experiments suggest that *CDK2AP1* behaves as a growth suppressor gene. The aim of our study was to measure the mRNA levels of *CDK2AP1* in human breast cancer and analyse the relationships between *CDK2AP1* levels and clinicopathological parameters and establish whether *CDK2AP1* behaves as a tumour suppressor. To our knowledge, this is the first study in the literature to examine the *CDK2AP1* expression levels in breast cancer.

Materials and Methods

Patients and samples. The breast cancer tissue and associated non-cancerous tissue (ANCT) samples studied in this cohort were originally collected from 1990 to 1994 under appropriate contemporaneous institution guidelines and ethical approvals.

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. Anonymous clinicopathological data were collected from the patient charts, and was collated in a secure air-gapped database. At the time of surgical excision, the patients were not subjected to any neoadjuvant treatment, as it was not a part of local guidelines at the time. In other words, the expression levels of *CDK2AP1* seen in this study would be reflective of baseline measurements without being confounded by the potential effects of chemotherapy. A total of 103 breast cancer tissues and 26 ANCTs were included in this study. Immediately after surgical excision, a tumour sample was taken from the tumour area while another was taken from ANCT within 2 cm from the tumour area, without affecting the assessment of tumour margins.

Tissue processing. The actual tissue processing, RNA extraction and cDNA synthesis were carried out at the time of original sample collection.

The quality of samples, thus, prepared was assessed by measuring β -actin mRNA expression, and normalising to 250 ng of RNA.

RNA extraction and cDNA synthesis. Reverse transcription was carried out using a reverse transcription kit (AbGene) with an 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.

RNA extraction kits and reverse transcription kits were obtained from Sigma-Aldrich Ltd (Poole, Dorset, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Sigma-Aldrich. Custom-made hot-start Master-mix for quantitative PCR was obtained from Abgene (Surrey, UK) (9, 10).

Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut at a thickness of 5-10 mm and kept for routine histological analysis. Additional 15-20 sections were mixed and homogenized using a hand-held homogenizer in ice-cold RNA extraction solution. The concentration of RNA was determined using UV spectrophotometry. Reverse transcription was carried out using a reverse transcription kit with an anchored oligo (dT) primer supplied by Abgene. The quality of cDNA was verified using β -actin primers (Appendix 1).

Quantitative analysis (12, 13). The quantitative PCR analyses were performed as follows. Transcripts of cDNA library were determined using real-time quantitative PCR based on Amplifluor Technology (12). The PCR primers were designed using Beacon Designer software, 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 I.

The levels of each transcript were generated from a standard plasmid which contained the specific DNA sequence that was simultaneously amplified within the samples.

With every run of the PCR, negative and positive controls were employed: Water was used as a negative control, whilst cDNA synthesised from a mixture of cancerous breast tissue was used as a positive control. In addition, a row of wells was seeded with known concentrations of podoplanin cDNA to serve as standards. Podoplanin is a protein sequence which occurs rarely in nature. This makes it ideal for calibration of PCR as that makes errors due to contamination unlikely.

Levels of *CDK2AP1* expression were then normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression, already measured in these specimens in order to correct for differing amounts of epithelial tissue between samples. *GAPDH* transcripts were quantified using primers detailed in Table I.

Statistical analysis. Statistical analysis was carried out using Minitab (14.1, Pennsylvania State University, USA) using a custom-written macro (Stat 2005.mtw). To assess the significance of associations between *CDK2AP1* levels and clinicopathological parameters, the two-sample *t*-test was used and a 95% confidence interval was calculated. *p*-Values less than 0.05 were considered significant, whereas *p*-values between 0.05 and 0.10 were considered marginally significant. Kaplan–Meier survival curves were used to analyse the association of *CDK2AP1* transcript levels with clinical outcome. *CDK2AP1* transcript levels in breast cancer specimens were compared to those of normal background tissues and analysed according to conventional pathological parameters and clinical outcome over a 10-year follow-up period. In each case, the true copy number was used for statistical analysis and hence samples were not classified as positive or negative.

Results

A total of 134 samples were analysed (Table II). Five samples were excluded because results from the analysis were uninterpretable, possibly due to sample contamination. *GAPDH*, a widely known ‘housekeeping gene’ (14), was used as a marker to normalise the *CDK2AP1* data. *CDK2AP1* expression was found in benign as well as cancerous specimens.

The mean normalised level of *CDK2AP1* was found to be 38-times higher in ANCT, but this result was non-significant ($p=0.33$). Twenty-four ANCT and 29 tumour samples were paired with each other to control for confounding factors; *CDK2AP1* expression in ANCT was 17-times higher than in tumour tissue, although this result was also not statistically significant ($p=0.35$).

We then examined the correlation between *CDK2AP1* expression and grade of breast cancer. A positive trend between *CDK2AP1* expression and grades 1-3 was observed; however, there was no statistically significant association. Further research is required to determine the relationship between tumour grade and *CDK2AP1* expression because this association potentially contradicts our hypothesis that

Table II. mRNA expression of cyclin-dependent kinase 2-associated protein 1 (*CDK2AP1*) in patients with breast cancer according to subgroup.

Parameter	No.	<i>CDK2AP1</i> expression (mean±SD)
<i>CDK2AP1</i> expression		
All tissue	134	9752±112309
ANCT	26	233±1163
Tumour tissue	103	9.06±26.61
Tumour grade		
1	16	0.949±2.849
2	33	5.29±16.04
3	52	8.35±35.18
TNM stage		
1	54	2.58±7.64
2	32	9.76±37.58
3	7	0.00000±0.00001
4	4	0.00160±0.00194
Recurrence/survival		
Alive with no recurrence	72	6.65±30.23
Alive with local recurrence	7	13.2±29.9
Alive with distant recurrence	5	5.92±13.24
Died of breast cancer	13	1.82±6.41
Histological type		
All	103	6.06±26.61
Ductal	81	6.86±29.72
Lobular	11	2.71±8.97
Mucinous	3	7.78±13.31
Medullary	2	5.52±7.80
Tubular	1	N/A
Other	6	5.73±9.60
ER status		
ER-negative	62	7.53±32.42
ERα-positive	31	5.07±15.94
ERβ-negative	73	7.95±31.34
ERβ-positive	22	1.98±5.43

ANCT: Associated non-cancerous tissue; ER: Estrogen receptor; N/A: not available.

CDK2AP1 has a tumour-suppressive function. Some of the results approached statistical significance: Grade 1 compared to grade 2 ($p=0.14$), grade 1 compared to grade 3 ($p=0.14$), and grade 1 compared to grades 2 and 3 combined ($p=0.059$).

Next, we examined the correlation present between TNM stage and *CDK2AP1* expression. Interestingly, there was a positive correlation between *CDK2AP1* expression and TNM1 and 2, but an overall negative correlation for TNM1 and 3, TNM1 and TNM4, TNM2 and 3, TNM2 and 4, and TNM3 and 4. There were no statistically significant results involving TNM2. The decrease in *CDK2AP1* expression between TNM1 and TNM3 was statistically significant ($p=0.016$), as was the decrease in *CDK2AP1* expression between TNM1 and TNM4 ($p=0.016$).

We also examined the association between clinical outcome and *CDK2AP1* expression over the 10-year follow-up period. Patients who were disease-free at 10 years had more than three times the amount of *CDK2AP1* expression as patients who had died of breast cancer by 10 years; however, this did not reach statistical significance. There was no significant difference in *CDK2AP1* mRNA levels in samples from patients who developed metastasis, local recurrence, or died of breast cancer when compared to those who were disease-free for 10 years (Kaplan–Meier analysis, $p=0.85$). Figure 1 shows the Kaplan–Meier curve for overall survival.

We found no statistically significant relationships between *CDK2AP1* expression and estrogen receptor status.

Finally, we investigated the correlation between *CDK2AP1* and *CDK2AP2* in 115 samples and observed a statistically significant positive correlation (correlation coefficient: $r^2=0.187$, $p=0.0453$).

Discussion

As far as we are aware, this is the first study to examine the mRNA expression of *CDK2AP1* in human breast cancer. Our findings suggest that *CDK2AP1* is likely to have a tumour-suppressive function and its mRNA expression might act as a biomarker of disease progression and as a potential therapeutic target in human breast cancer.

Our observations are in accordance with a wealth of laboratory studies supporting the notion that *CDK2AP1* behaves as a tumour-suppressor gene in a variety of tumours (12–19). Zou *et al.* reported that *CDK2AP1* inhibited the proliferation of breast cancer cells and the *in vivo* growth of tumour cells (15). In their experiment, *CDK2AP1* expression was significantly down-regulated in breast cancer cases when compared to corresponding non-tumorous tissues. In immunodeficient mice, its overexpression led to G_0/G_1 phase arrest and inhibited *in vivo* tumour growth. *CDK2AP1* knockdown resulted in enhanced colony formation and cell growth. It also inhibited the growth of breast cancer cells through regulation of the cell cycle and increased *in vitro* and *in vivo* sensitivity to docetaxel (16).

In an *in vivo* SCXC floor of mouth xenograft mouse model, tumour growth was inhibited by *CDK2AP1* gene therapy (17). *CDK2AP1* gene therapy, when compared to controls, significantly induced multiple antitumour effects: it reduced the size and weight of tumours, increased inhibition of tumour growth rate post therapy, increased *CDK2AP1* expression, increased terminal nucleotidyl transferase-mediated nick- end labelling, increased morphology-based apoptotic indices, and reduced Ki-67 cell proliferation indices.

CDK2AP1 has also been shown to mediate DNA damage responses induced by cisplatin and enhance chemosensitivity (18). Knockout clones displayed resistance to cisplatin

Figure 1. Kaplan–Meier analysis of overall survival according to disease-free status [log-rank test (Mantel–Cox): $p=0.872$].

treatment, significant post-treatment reduction in apoptosis, and sustained CDK2 kinase activity despite cisplatin treatment. This finding suggests *CDK2AP1* could potentially be used as a therapeutic agent.

Epithelial–mesenchymal transition (EMT) has been associated multiple times with breast cancer development (19), (20), specifically the basal-like phenotype (21). By promoting twist family bHLH transcription factor 2 (TWIST2), *CDK2AP1* induced EMT of hamster cheek pouch carcinoma 1 cells (22). EMT cells, however, interestingly had an increased invasive and a reduced metastatic phenotype. To complete spontaneous metastasis, EMT and non-EMT cells were shown to cooperate. The authors of this investigation acknowledged that the failure to metastasise may simply be due to the intrinsic properties of *CDK2AP1* as a growth suppressor. This may have an impact on the decision to use *CDK2AP1* in any therapeutic context.

Our observations are consistent with these reports and lend further support to the notion that *CDK2AP1* has a tumour-suppressive function in human breast cancer with potential clinical applications as a marker of disease progression and as a therapeutic indicator.

The strength of our study lies in the use of robust RT-PCR methodology to analyse *CDK2AP1* mRNA expression in a cohort of patients with breast cancer with long-term follow-up.

We previously demonstrated that *CDK2AP2* gene has a tumour-suppressive function (6), therefore our observed statistically significant correlation between *CDK2AP1* and *CDK2AP2* is not surprising and requires further investigation.

The limitations of our investigation included the use of ANCT to provide 'normal tissue' for comparison. Ideally, such tissue should be obtained from patients without breast cancer to avoid any associated molecular changes which may be found within ANCT. Although the follow-up period was sufficient for analysis of clinical outcome, the small sample size might have contributed to the lack of a statistically significant relationship of *CDK2AP1* with survival. It is possible that analysis of a larger cohort could influence several results which approached but failed to reach statistical significance. Finally, in addition to the measurement of *CDK2AP1* mRNA transcript levels, quantification of protein expression should be performed to ensure concordance.

Conclusion

To our knowledge, we are the first group to examine *CDK2AP1* mRNA expression in human breast cancer and demonstrate that its level decreases with disease progression.

Further research is required to confirm the role of *CDK2AP1* as a tumour suppressor in human breast cancer. New immunohistochemical studies, *in vitro* and *in vivo* experiments, and larger validation studies are required to confirm the impact of *CDK2AP1* expression on human breast cancer pathogenesis and its value in prognosis and gene therapy.

If our observations are confirmed by larger validation studies, *CDK2AP1* might become a valuable biomarker of disease progression. Its artificial expression might become a novel therapeutic strategy in human breast cancer.

Conflicts of Interest

The Authors declare that they have no competing interests in regard to this study.

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