The mRNA Expression of Inhibitors of DNA Binding-1 and -2 Is Associated with Advanced Tumour Stage and Adverse Clinical Outcome in Human Breast Cancer

UMAR WAZIR[1,2], WEN G. JIANG[3], ANUP K. SHARMA[2], ROBERT F. NEWBOLD[1] and KEFAH MOKBEL[1,2]

[1]*The London Breast Institute, Princess Grace Hospital, London, UK;*
[2]*Department of Breast Surgery, St. George's Hospital and Medical School, University of London, London, U.K;*
[3]*Metastasis and Angiogenesis Research Group, Department of Surgery, School of Medicine, Cardiff University, Cardiff, Wales, U.K.*

Abstract. *Inhibitors of DNA binding (ID) are known to have a role in embryogenesis and oncogenesis. In this study, we analyzed the role of ID1 and ID2 in breast cancer, by assessing associations of mRNA expression with clinicopathological parameters. Materials and Methods: Breast cancer tissues (n=152) and adjacent normal tissues (n=31) underwent reverse transcription and quantitative- polymerase chain reaction (qPCR). Transcript levels were correlated with clinico-pathological data. Results: Patients who were disease–free had significantly lower ID1 mRNA expression than all other patients (p=0.0033). Higher expression was associated with worse disease-free (p=0.001) and overall survival (p=0.02). ID2 expression was directly associated with the Nottingham Prognostic Index (NPI) (NPI 2 vs. 3; p=0.0062) and worsening clinical outcome (disease-free vs. mortality: p=0.0004), and with worse disease-free (p=0.01) and overall survival (p=0.005). Conclusion: Our findings are suggestive of a role for ID1 and ID2 in human breast cancer as possible prognostic markers and therapeutic targets meriting further validating investigations, by immunohistochemistry and mechanistic studies.*

The basic helix-loop-helix (HLH) proteins are highly-conserved proteins which mediate DNA–protein interactions. They have a key role in embryogenesis and cell differentiation, and encourage cell proliferation and differentiation of pluripotent cells.

Inhibitors of DNA-binding (ID1 to ID4) are a sub-type of HLH proteins, which lack the DNA-binding domain. They inhibit the actions of basic HLH proteins by binding to them and thus by blocking their interactions with DNA. ID proteins have been found to have a role in embryogenesis, cell proliferation, haematopoiesis, and carcinogenesis (1, 2).

In this study we examined the associations between ID1, ID2 and ID3 expressions and clinicopathological parameters of human breast cancer in a cohort with a decade of follow-up.

Correspondence to: Professor Kefah Mokbel, London Breast Institute, the Princess Grace Hospital, 45 Nottingham Place, London W1U 5NY, U.K. E-mail: kefahmokbel@hotmail.com

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Materials and Methods

Samples. Tissue samples were collected after informed consent with ethical approval as per contemporaneous institutional guidelines (Bro Taf Health Authority ethics approval numbers 01/4303 and 01/4046). 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=152) and normal background tissues (n=31) were collected and stored at −80˚C in liquid nitrogen until the commencement of this study. This cohort has been the subject of a number of completed and on-going studies (3-5).

All the patients were treated according to local guidelines, following discussions in multidisciplinary meetings. Patients undergoing breast-conserving surgery also underwent radiotherapy. Patients with hormone-sensitive disease 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.

RNA extraction kits and reverse transcription kits were obtained from AbGene Ltd. (Epsom, Surrey, UK). PCR primers for *ID1*, *ID2* and *ID3* were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Invitrogen Ltd. (Paisley, UK). Custom made hot-start Master Mix for quantitative PCR was from AbGene (6).

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

Table I. *Clinical data describing the patient cohort.*

Parameter	Category	Number
Node status	Node positive	53
	Node negative	99
Tumour grade	1	20
	2	39
	3	54
Tumour type	Ductal	89
	Lobular	12
	Medullary	2
	Tubular	1
	Mucinous	4
	Other	7
TNM staging	1	61
	2	37
	3	7
	4	4
Clinical outcome	Disease-free	81
	With local recurrence	7
	Alive with metastasis	5
	Died of breast cancer	14

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 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'-ATGATATCGCCGCGCTCGTC-3' and 5'-CGCTCGGTGAGGATCTTCA-3') (6).

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., Palo 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 expressions of *ID1*, *ID2* and *ID3* were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

With every run of the PCR, a negative and positive control were employed, using a known cDNA sequence (podoplannin) (6).

Statistical analysis. Analysis of the data was performed using the Minitab 12 statistical software package (Minitab Ltd., Coventry, UK.) with 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')
ID1 F1	tcaacggcgagatcag
ID1 ZR1	actgaacctgaccgtacagatcgtccgcaggaa
ID2 F1	caacacggatatcagcatc
ID2 ZR1	actgaacctgaccgtacaacagtgctttgctgtcattt
ID3 F1	gcgtcatcgactacattctc
ID3 ZR1	actgaacctgaccgtacagtcgttggagatgacaagtt
Beta-actin forward	atgatatcgccgcgctcgtc
Beta-actin reverse	cgctcggtgaggatcttca

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

ID1 mRNA expression was found to be increased in breast cancer samples. Its expression was significantly associated with adverse clinical outcomes. This was highly significant when comparing patients who had no recurrences over a decade of follow-up with those with disease-related mortality (p=0.0169), and when comparing patients who were disease-free with all other patients (p=0.0033) (Table III).

A similar trend was noted between *ID1* mRNA expression and tumour grade, TNM stage, and NPI. However, these trends did not achieve statistical significance.

The Kaplan–Meier plot analysis demonstrated that patients with higher *ID1* mRNA expression had reduced disease-free (p=0.001) and overall (p=0.02) survival (Figures 1 and 2).

ID2 expression was also found to be directly associated with breast cancer. *ID2* expression was significantly increased with higher NPI scores (NPI 2 *vs*. 3; p=0.0062).

Mean and median *ID2* copy numbers were also significantly increased with advanced disease. This attained highest significance when comparing patients who were disease-free during follow-up with those with disease-related mortality (p=0.0004).

A similar, albeit insignificant, association was seen between *ID2* expression, tumour grade and TNM stage (Table IV).

This was further illustrated by the Kaplan–Meier plot analysis, in which higher *ID2* mRNA expression was associated with worse disease-free (p=0.01) and overall (p=0.005) survival (Figures 3 and 4).

ID3 mRNA expression was also studied. However, no statistically significant associations were found in relation to the clinicopathological parameters of human breast cancer in this cohort.

Table III. *Comparison of Inhibitor of DNA binding-1 (ID1) mRNA expression levels (expressed as median copy numbers normalized against GAPDH) in subgroups within the cohort.*

Patient and tumour characteristic	Median(s)	95% Confidence interval	*p*-Value
Survival			
DF *vs*. LR	0.21 *vs*. 12.14	–37.22 to –1.08	0.0109
DF *vs*. DR	0.21 *vs*. 0.01	–3.920 to 0.619	0.8319
DF *vs*. D	0.21 *vs*. 5.76	–11.40 to –0.00	0.0169
DF *vs*. LR/DR/D	0.21 *vs*. 3.86	–10.95 to –0.02	0.0033

DF: Disease-free survival, LR: local disease recurrence, DR: distant disease recurrence, D: death from breast cancer.

Table IV. *Comparison of Inhibitor of DNA binding-2 (ID2) mRNA expression levels (expressed as median copy numbers normalized against GAPDH) in subgroups within the cohort.*

Patient and tumour characteristic	Median(s)	95% Confidence interval	*p*-Value
Tumour grade			
1 *vs*. 2	105.7 *vs*. 15.1	–130.7 to 102.5	0.4469
1 *vs*. 3	105.7 *vs*. 175.2	–241.8 to 77.1	0.9466
2 *vs*. 3	15.1 *vs*. 175.2	–195.6 to 4.5	0.3190
NPI			
1 *vs*. 2	175.2 *vs*. 8.8	–0.1 to 230.5	0.0546
1 *vs*. 3	175.2 *vs*. 1723.9	–2299.2 to –0.0	0.0493
2 *vs*. 3	8.8 *vs*. 1723.9	–2314.8 to –6.0	0.0062
TNM			
1 *vs*. 2	83.8 *vs*. 149.1	–110.3 to 44.6	0.9883
1 *vs*. 3	83.8 *vs*. 420.2	–1350.0 to 289.3	0.6644
1 *vs*. 4	83.8 *vs*. 884.1	–2368.5 to 616.9	0.6329
2 *vs*. 3	149.1 *vs*. 420.2	–1350.3 to 258.0	0.7241
2 *vs*. 4	149.1 *vs*. 884.1	–2667.3 to 1186.2	0.5531
3 *vs*. 4	420.2 *vs*. 884.1	–3439.5 to 3278.4	0.9247
Survival			
DF *vs*. LR	40.2 *vs*. 13.5	–618.7 to 148.8	0.7636
DF *vs*. DR	40.2 *vs*. 289.4	–326.1 to 215.1	0.4383
DF *vs*. D	40.2 *vs*. 2038.5	–5631.0 to –252.1	0.0004
DF *vs*. LR/DR/D	40.2 *vs*. 713.4	–1263.0 to –9.3	0.0038

NPI: Nottingham Prognostic Index, TNM: clinical stage according to tumour size, Nodal status and presence of distant Metastases, DF: disease-free, LR: local disease recurrence, DR: distant disease recurrence, D: death from breast cancer.

Discussion

The basic HLH transcription factors have been implicated in the regulation of differentiation and growth of pluripotent stem cells. They are characterised by a DNA-binding region specific for a sequence termed the E-box, and motifs for interaction with other proteins. They are ubiquitous and evolutionarily-conserved proteins. They facilitate differentiation of cells by inducing transcription of cell differentiation factors. In addition, they induce cell-cycle arrest *via* cyclin-dependent kinase inhibitors (CKIs), such as p15, p16 and p21 (1, 7).

The ID proteins were identified in the early 1990s as a class of HLH proteins lacking the basic DNA-binding region. It was initially postulated that they inhibit the actions of basic HLH proteins by sequestering them from interactions with the genome. ID and basic HLH transcription factors are believed to serve as counterpoints in the formation of the basic body plan during embryogenesis (2, 8).

This role has been confirmed by *in vitro* and *in vivo* evidence. ID proteins 1, 2 and 3 have been found to be ubiquitously expressed in mammalian tissues, whilst ID4 is largely expressed in the nervous system, testes and fatty tissue. Increased expression of ID proteins has been associated with a number of neoplasias, including carcinomas of the colon (9), lungs (10), breast (11), and pancreas (12).

Studies in MDA-MB-231 cells by Tobin *et al*. found that silencing of cyclin-D1 was associated with increased migration, and was likely mediated by ID1. A meta-analysis of gene expressions in breast cancer samples suggested an association between reduced disease-free survival, reduced cyclin-D1 and increased *ID1* expression (13).

Lasorella *et al*. determined that ID2 interacted with the retinoblastoma protein (pRb), p107 and p130. Furthermore, increased ID2 expression was associated with reversal of p16– and p21–induced cell-cycle arrest. In addition, cyclin-D1 expression was found to be suppressed by ID2 (14).

Counterintuitively, in a study of breast cancer specimens using immunohistochemistry in a cohort of 114 patients, Stighall *et al*. found an association between high cytoplasmic staining for ID2 and favourable prognosis. In addition, ectopic ID2 expression in MDA-MB-468 breast cancer cells was found to increase cell invasiveness and proliferation (15).

Itahana *et al*. found a similar association between ID2 expression and invasiveness and in a study of MDA-MB231 cells. However, a relationship with proliferation was not found in this instance (16).

In contrast, in a more recent study using immunohistochemistry on specimens from patients with non-small cell lung cancer, Rollin *et al*. identified ID2 as a marker of unfavourable prognosis for those with poorly-differentiated tumours (17).

In addition, a study on MCF-7 breast cancer cells and SKOV-3 ovarian cancer cells suggested an association between ID2 expression and increased cell migration (18).

The role of stem cells and tumour microenvironment in the biology of solid tumours, including breast cancer, has been the subject of recent research (19, 20). Recent studies in colonic carcinoma purport that tumour cells are replenished

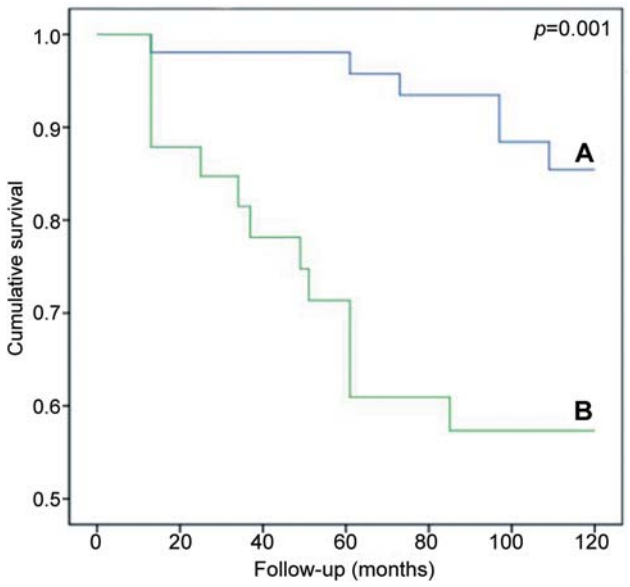

Figure 1. *Disease-free survival curve according to mRNA expression of ID1. Curve A (lower transcription group) and curve B (higher transcription group) are defined by the moderate risk group by the Nottingham prognosis index (NPI) serving as the dividing line.*

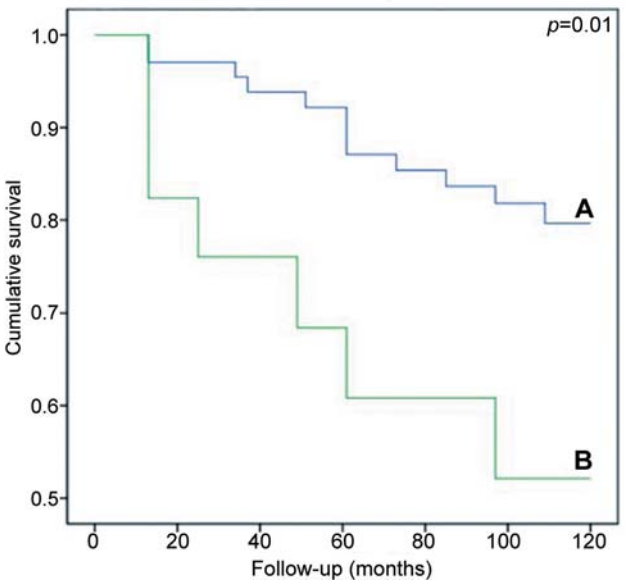

Figure 3. *Disease-free survival curve according to mRNA expression of ID2. Curve A (lower transcription group) and curve B (higher transcription group) are defined by the moderate risk group by the Nottingham prognosis index (NPI) serving as the dividing line.*

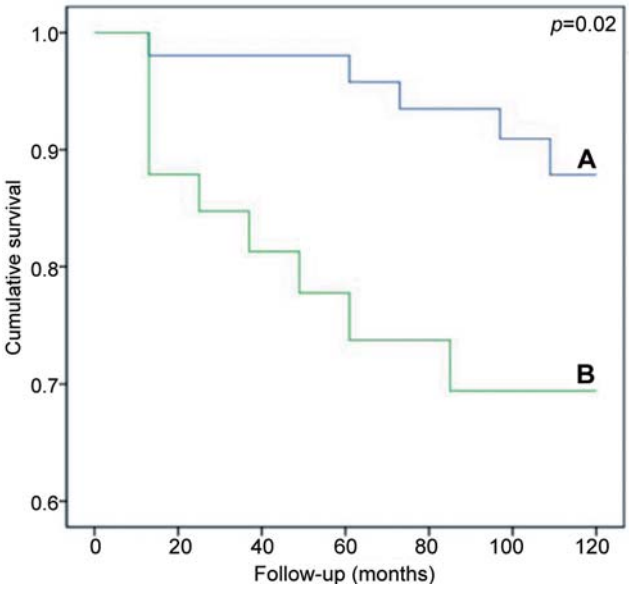

Figure 2. *Overall survival curve according to mRNA expression of ID1. Curve A (lower transcription group) and curve B (higher transcription group) are defined by the moderate risk group by the Nottingham prognosis index (NPI) serving as the dividing line.*

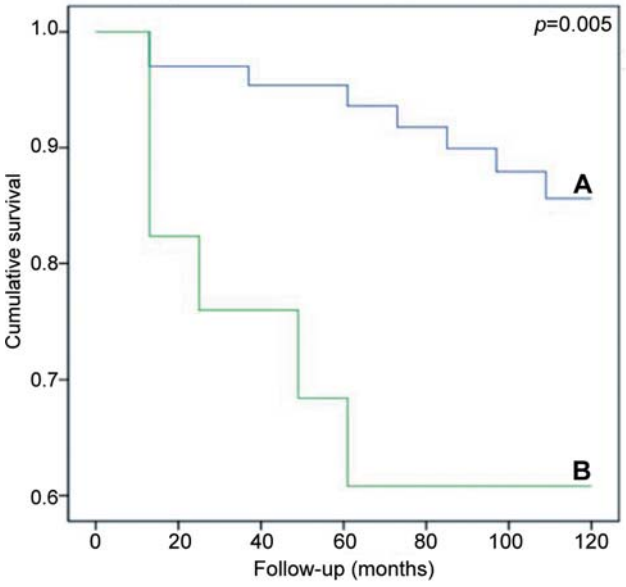

Figure 4. *Overall survival curve according to mRNA expression of ID2. Curve A (lower transcription group) and curve B (higher transcription group) are defined by moderate risk group by the Nottingham prognosis index (NPI) serving as the dividing line.*

by a sub-population characterized as initiator cells. O'Brien *et al*. has suggested that ID1 and ID3 play a role in colonic carcinoma cell renewal *via* p21-mediated cell-cycle arrest (9).

Conclusion

The role of ID1 is well-established in human breast cancer, and our findings are in keeping with those of other authors. However, to our knowledge, ours is the first study to suggest an association of *ID2* mRNA expression and clinicopathological parameters of human breast cancer. Furthermore, our results are based upon well-tested and robust quantitative PCR, performed on a cohort with at least a decade of follow-up.

Recent research in breast cancer suggests a major role for stem cells in the maintenance of an oncogenic tumour microenvironment, which in turn may lead to the recruitment of additional cells to the pathology (19). In view of the previously cited literature, our results potentially suggest a place for ID1 and ID2 in the maintenance of a cancer-initiator cell population in poorly-differentiated human breast cancer. We hope that our findings provide clinical evidence for further research in the role of cell-cycle regulatory proteins and of stem cells in human breast cancer.

Acknowledgements

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