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Disregulation of Urokinase Plasminogen Activator Gene in Breast Cancer

A.L. Hubbard, J. Lauder, R.A. Hawkins and T.J. Anderson

Disregulation of urokinase plasminogen activator (uPA) was assessed in 134 breast cancer specimens. Overexpression of uPA was determined by immunohistochemistry using the specific monoclonal antibody, #394. Gene amplification was assessed by differential polymerase chain reaction, using primers designed to amplify a 111 bp segment of the uPA gene. Overexpression of uPA was detected in 33% of breast cancers, including 4 of 21 *in situ* carcinomas, 7 of 14 lobular and 2 of 10 tubular carcinomas. Overexpression of uPA did not correlate with the presence or absence of oestrogen receptors. uPA gene amplification was not detected in any cancer. We conclude that uPA gene amplification is not a major mechanism instigating uPA overexpression in breast cancer, and that overexpression is likely to be controlled by other mechanisms.

Key words: breast carcinoma, urokinase plasminogen activator, gene amplification, gene overexpression
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INTRODUCTION

OVEREXPRESSION OF the serine protease,urokinase plasminogen activator (uPA) in epithelial cells has been linked to aggressive behaviour in breast cancer [1, 2]. The biochemical action of the enzyme may promote metastasis by degradation of proteins, such as collagen, in the extracellular tumour stroma [3]. The conversion of plasminogen to plasmin by uPA is thought to be a critical step in this degradation [4]. Previous studies of breast cancers with immunohistochemistry using a monoclonal antibody directed against human uPA have shown overexpression

of uPA in all of 115 cancers tested [1]. Analysis of uPA antigen by ELISA in the cytosolic fraction of these cancers indicated that they contained, on average, 11 times more uPA than normal breast tissue [1]. High concentrations of uPA in breast cancer tissue may be an independent prognostic factor in predicting early relapse [1, 5, 6, 7].

The mechanisms of overexpression of uPA in breast cancer are not clear, and may involve disregulated promoter and/or suppressor activity, or fundamental DNA changes such as gene amplification. The regulation of the concentration of uPA in the cytoplasm of cells is complex; it can be influenced by a number of cell signals such as hormones [8], phorbol esters [9], growth factors [10], and cytokines [11]. Another possible mechanism for overexpression is gene amplification. Overexpression associated with gene amplification has been demonstrated in breast cancer for some oncogenes, such as c-*erb* B2 and c-*myc* [12–14], and have shown that these genes can be disregulated by more than one mechanism. We examined the possibility that overexpression of uPA could be due, in some cases, to uPA gene amplification.

MATERIALS AND METHODS

Breast tissues

Samples of tissue were collected from 134 breast cancers at routine surgical operations, which included mastectomy and open biopsy for both palpable and non-palpable lesions; all were primary cancers which had not been included in neo-adjuvant treatment studies [15]. Samples were restricted to the age group 50–65 years and were collected from January 1988 to May 1990. They were fixed in methacarn (6 : 3 : 1, methanol : choloroform : acetic acid) overnight at 4°C, processed according to routine methods and embedded in paraffin. Control tissue was obtained from breast tissue distant from the lesion site or from non-cancer bearing breasts. Pathological characterisation was taken from overall evaluation of material used for routine diagnosis and assessed according to standard criteria for histological type, grade, and node status [16]. This included an evaluation of a 4 μm section immediately adjacent to the tissue used for differential PCR (see below) to confirm its histopathological nature. The specimen's cellularity, namely the proportion of cancer cells present, and ploidy status was assessed according to published methods [17].

Oestrogen receptors

Oestrogen receptors were measured from tissue collected, frozen and stored at −196°C at the time of operation. A radioligand-binding assay using dextran-coated charcoal was used according to the method of Hawkins and colleagues [18]. The concentration of oestrogen receptors in each cancer sample was expressed as a concentration of protein, with values of >20 fmol/mg protein considered clinically significant.

Immunohistochemistry

Expression of uPA protein was examined with a murine monoclonal antibody, #394, claimed as specific for human urokinase plasminogen activator (American Diagnostica Inc. New York, NY, U.S.A.). Four micron paraffin sections of all cancers were dried at 56°C overnight. The sections were dewaxed in xylene and rehydrated to water. Endogenous peroxidase was blocked by exposure to 1% hydrogen peroxide in methanol for 15 min before staining. The sections were washed in Tris-buffered saline (TBS pH 7.6), followed by a 10 min incubation with normal rabbit serum (Scottish Antibody Production Unit, Carluke, U.K.) diluted 1 : 5 with TBS. The primary antibody was applied at a 1 : 100 dilution in normal rabbit serum/TBS and incubated at room temperature for 30 min. Normal rabbit serum was used in place of the primary antibody as a negative control. An ABComplex method was carried out according to manufacturers instructions (Dako K355). The sections were lightly counterstained in haematoxylin. To score positive, indicating uPA expression, cancer cells had to show weak, moderate or strong brown staining of cytoplasm above normal stromal components of the tissue section, excluding macrophages. The assessment of uPA expression was based on a single tissue section from each specimen and was jointly assessed by TJA and ALH. A positive control, a cancer previously identified as showing strong staining with #394, and a negative control were incorporated in each set of sections for staining. Some variation in staining intensity was noted in positive control sections. As the titre of antibody #394 dropped from 1 : 100 to 1 : 10 during overnight storage after reconstitution of the lyophilised preparation, fresh dilutions of #394 were prepared for each batch of slides. This occurred with three separate batches of antibody.

Primers and the differential polymerase chain reaction

Primers for differential PCR for uPA were designed from the human gene sequence published elsewhere [19]. The primer sequence for the sense strand was 5′ CAGTTTACCCTCACCCTGGA 3′ (1631–1650 bp), and the antisense strand 5′ AGCCAACTGTTGTAGGGGTG 3′ (1757–1738 bp). PCR using these primers yields a 111 bp product from the intron 2-exon 3 boundary of the published sequence. The single copy reference gene used in dPCR was a 150 bp sequence of human interferon gamma [20]. For dPCR, four 10 μm sections of fixed, paraffin-embedded tissue were added to 100 μl of lysis buffer (50 mM Tris HCl, pH 8.4, 1 mM EDTA, 0.5% Tween 20) and boiled for 8 min [21]. Differential PCR was performed on a Techne PHC 3 thermal cycler incorporating 5 μl of prepared lysed paraffin section or 200 ng of control DNA (human placental), 0.25 μM each primer, 200 mM dNTPs (Pharmacia), ×1 Taq polymerase buffer (Northumbria Biotechnology Limited NBL), 1 unit of Taq polymerase (NBL) and 3 μCi of ^{32}P CTP (New England Nuclear). Cycling parameters were one cycle of 94°C for 5 min, 50°C for 1 min, 70°C for 1 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 70°C for 1 min and one cycle of 94°C for 1 min, 50°C for 1 min, 70°C for 5 min. Duplicate PCR products were separated by size on 2% agarose gels, and stained with ethidium bromide. Visible bands were excised, finely chopped and added to 5 ml of Optiphase-safe scintillation fluid. Radioactivity was assessed as counts per minute (CPM) on a Beckman scintillation counter. Results from dPCR are expressed as ratio values and were calculated by averaging the CPM from duplicate gel tracks and subtracting the average experimental blank. For uPA, a correction factor of 1.04 was applied to compensate for differences in dCTP content between amplicons IFNG150 (69 C bases) and uPA (66 C bases). To ascertain the relative copy number of uPA to the reference gene, the corrected average CPM for uPA was divided by the average CPM for IFNG150, giving in each case a result expressed as a ratio value.

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This method has been previously validated for use of fixed breast tissues and shown to detect amplifications of the oncogenes c-*erb* B2, EGFR and N-*ras* [17, 20].

RESULTS

Immunohistochemistry

Immunohistochemistry for expression of uPA protein was performed on 134 breast cancer specimens. Overexpression of uPA protein was observed in 40 of 113 (35%) invasive cancers and in 4 of 21 (19%) *in situ* cancers, (total cancers 33%). Staining was concentrated in the cytoplasm of the cells, and usually present evenly throughout the cancer cell population (Figure 1). Staining of the cell membrane was present in some positive cancers, however, this was always in association with cytoplasmic staining. In some cases, weak staining was observed in normal stroma and infiltrating lymphocytes, but this was always in association with positively stained cancer cells. Strong staining was observed in cells identified as macrophages in a small number of cases, and if staining was present only in macrophages, specimens were considered negative.

Table 1 summarises the association between uPA protein overexpression and features of the cancers studied. uPA expression was more common in invasive cancers, and within that group was more frequent in oestrogen receptor negative cancers. "Positive" staining was present in similar proportions in each of the ploidy groups, diploid (36%), aneuploid (43%) and tetraploid (38%). We found no correlation between size of cancer or node status with overexpression. Grade 1 cancers showed a lower frequency of overexpression (24%) than grade 2 or 3 (38%), but these differences were not significant.

Overexpression was not confined to any particular histological type of cancer, but noteworthy was the high proportion in lobular carcinomas (7 of 14), and overexpression was also present in some tubular carcinomas (2 of 10). Freqency of overexpression in each type of cancer is shown in Table 1.

Differential PCR

Figure 2 shows representative dPCR products obtained from three cancer specimens and a normal DNA control (derived from human placenta). Differential PCR ratio values were obtained for 134 cancer specimens and 33 control tissues (Table 2), and ranged from 0.52 to 1.54 (mean 0.9) in control tissues and 0.41–1.83 (mean 1.1) in cancer tissues. Ratio values of 2 or above have been considered to signify gene amplification

Table 1. Clinical features of the study population with uPA immunohistochemistry

	Number of cases	Percentage with positive immunohistochemistry
Invasive cancers	113	35
In situ cancers	21	19
Invasive cancers only		
Oestrogen receptor status		
<20 fmol/mg protein	32	41
≥20 fmol/mg protein	73	34
NK	8	
DNA ploidy		
Diploid	45	36
Aneuploid	28	43
Tetraploid	24	38
NK	6	
Cancer size		
1–10	10	50
11–20	38	24
21–30	41	44
31–40	12	33
>40	12	33
Lymph node status		
Positive	36	31
Negative	68	38
NK	9	
Cancer grade		
1	21	24
2	66	38
3	21	38
NK	5	
Cancer type		
DCI NST	86	35
LCI	14	50
TCI	10	20
OTH	3	33

Invasive cancer types are DCI NST, ductal carcinoma of no special type; LCI, lobular carcinoma; TCI, tubular carcinoma; and OTH, other special types; NK, not known.

in other studies [17]. There was no clear difference in range of values between invasive cancers (0.41–1.83) and *in situ* cancers (0.7–1.55). The distribution of ratio values was similar for immunohistochemistry-positive and -negative cancers (Figure 3). Cancers shown to be overexpressing uPA protein were distributed evenly over the range of dPCR values, indicating that cancers which overexpress uPA are not associated with high dPCR values. Differential PCR values were not influenced by specimen cellularity or DNA ploidy.

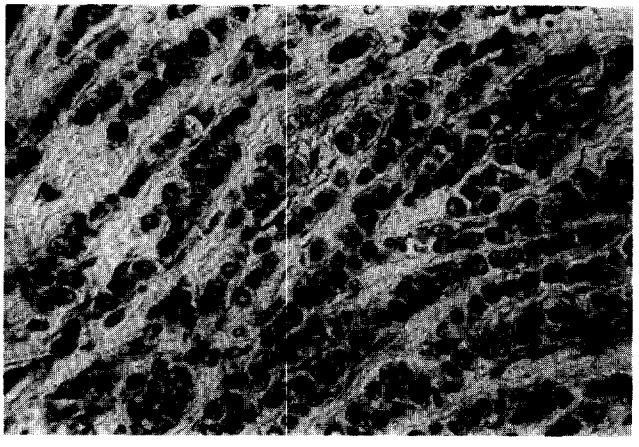

Figure 1. Four micron section of a lobular carcinoma showing strong positive staining with anti-uPA antibody (#394). Magnification × 160.

DISCUSSION

Using dPCR to study gene disregulation at the uPA locus, we found no evidence for amplification of this gene. We could not detect any relationship between overexpression of uPA protein and dPCR ratio values indicating increased gene copy number. We therefore conclude that uPA gene amplification is not a major influence on immunohistochemically detected overexpression of uPA in breast cancer. However, other interesting features were observed and are noteworthy of discussion.

Although no difference was detected between the ranges of dPCR ratio values for cancers and controls, cancers had a higher mean dPCR value. This could reflect a low level of chromosomal

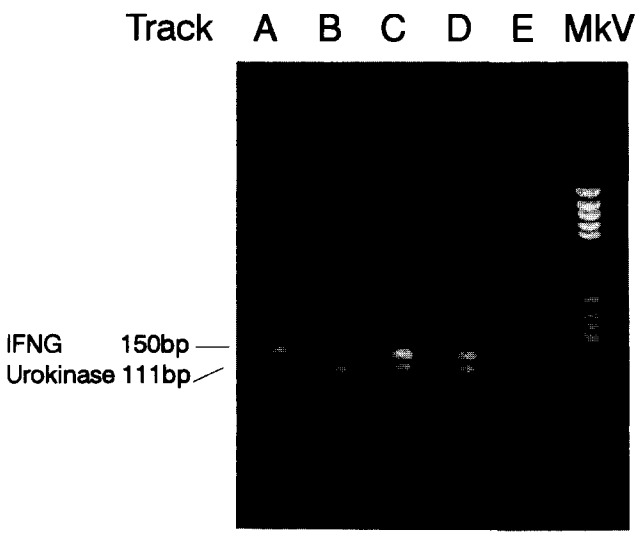

Figure 2. Differential PCR products from three different breast cancers (Tracks A, B and C) and one control placental DNA (Track D), separated on 2% 3 : 1 Nuseive/Seakem agarose gel. Track E contains the experimental negative control (PCR reaction minus template DNA). PCR products for IFNG (reference gene) and uPA are indicated at 150 bp and 111 bp, respectively.

Table 2. Ranges of dPCR ratio values found for invasive, in situ *breast cancers and control tissues*

	Number of cases	dPCR ratio range	Mean dPCR ratio value
Invasive cancers	113	0.41–1.83	1.12
In situ cancers	21	0.7 –1.55	1.18
Controls	33	0.52–1.54	0.90

instability, yet there was no association between high dPCR values and abnormalities of DNA ploidy. Furthermore, cases overexpressing uPA protein were not solely restricted to those with dPCR ratio values at the upper end of the normal range, indicating that overexpression was not due to minor chromosomal disregulation. The possibility that gene overexpression can lead to gene amplification has been suggested [22, 23], but has not been confirmed experimentally and seems unlikely to be true for the uPA gene.

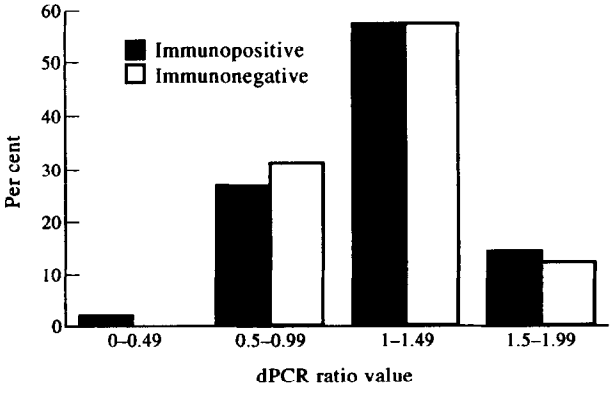

Figure 3. Range of dPCR values for immunohistochemistry positive and negative cancers.

We found uPA expression to be common in invasive lobular cancers. These cancers characteristically infiltrate through stromal elements as individual units in linear array, and uPA may play some part in this process. However, both invasive tubular carcinomas and *in situ* cancers also expressed uPA, yet these histological types are recognised for cell cohesion, differentiation and lack of metastasis [24, 25]. This suggests that overexpression of uPA can be an 'early' event and that additional factors are required for the development of invasion resulting in a poorer prognosis. The presence of uPA in the cytoplasm is probably only one of several possible factors involved in cancer cell dissemination. Examination of lung carcinoma cell lines have indicated that functionally active, receptor-bound, uPA is essential for tissue invasion [26]. Studies of uPA overexpression combined with assessment of cell surface receptors (uPAR) may illuminate the significance of overexpressing uPA, especially in cancers which have a favourable outcome.

Assessing the mechanisms accounting for overexpression of uPA in breast cancer is likely to be complex, and may involve factors which affect transcription, translation and post-transcriptional processing. Increased levels of specific uPA inhibitors, PAI-1, PAI-2 and protease nexin, in breast cancer cells have been shown to regulate cytoplasmic concentration of uPA [27]. Post-transcriptional activation mechanisms of uPA may also be disregulated in cancer cells [28]. Immunohistochemistry is a convenient method for demonstrating disregulated gene expression, and detection frequency is usually high, in the order of 88–100% of breast cancers [1, 29]. However, this study detected overexpression of uPA in only 33% of breast cancers, a frequency similar to another immunohistochemical study of breast cancer, although that study was based on frozen sections [30]. This recorded disparity in frequency of positivity is a cause for concern, but could be due to criteria for scoring positivity, differences in immunohistochemical protocols and antibodies, or both of these. Present requirement for staining above that of normal stroma was an excluding factor in only 6 cases, and is unlikely to be a major factor influencing the frequency of positivity. The instability of the antibody used here is a major factor causing concern and has been noted in other laboratories (M. Walch, personal communication) for unidentified reasons. There are alternative antibodies available for uPA which also detect high frequencies of expression in ELISA [7], but present experience suggests caution before drawing conclusions from immunohistochemistry alone.

Other features of our cancer population, such as size and ploidy, show no particular associations with uPA expression. The trends toward elevated uPA expression in grade 2 or 3 cancers and in negative lymph node status cancers were noted previously [6, 7]. Correlations of biochemical and immunochistochemical procedures generally have indicated that measured concentrations of uPA protein correlate with the strength of immunostaining, although individual cases can show major discrepancies [1]. This suggests that the relationship between immunohistochemical staining and detection of uPA protein in breast cancers is not strictly quantitative. Furthermore, the results of biochemical studies may be confounded by the presence of uPA-rich macrophages [31], indicating that a combination of immunohistochemistry and ELISA would be more informative than either technique alone. Comparability between studies is further confounded by differences in the experimental strategy and antibodies employed [1, 2, 6, 7, 30]. The concentrations of uPA in the cytoplasm of cancer cells is known to be affected by growth factors, phorbol esters, cytokines and

hormones [9–11, 32]. Studies of cell lines have shown that oestrogen complexed with its receptor can induce uPA synthesis [32]. We found no significant difference in the frequency of uPA protein overexpression in oestrogen receptor-positive and -negative cancers, suggesting that oestrogen is not a major influence in the complex regulatory mechanisms of uPA production.

In summary, our study has provided evidence that uPA is overexpressed in at least some breast cancers which may relate to gene disregulation, though not to gene amplification.

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