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ORIGINAL ARTICLE

The impact of delay in cryo-fixation on biomarkers of Src tyrosine kinase activity in human breast and bladder cancers

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Abstract Demonstration of pharmacodynamic activity of new, targeted cancer drugs in tumour tissue is potentially important in guiding early drug development. However, delays between tumour sampling and sample fixation may result in variability of pharmacodynamic biomarkers. The aim of this study, was to assess the impact of delays in fixation on biomarkers of Src kinase activity. A total of 20 patients with locally advanced breast cancer and 5 with early bladder cancer had multiple tissue samples taken which were fixed at documented time points up to 60 min after biopsy. These were examined to determine if the amount of Paxillin, phospho-Paxillin, phospho-focal adhesion kinase (FAK) and total phospho-Tyrosine changed over time, using a quantitative lysate immunoassay. In breast cancer, there was an increase in the amount of phospho-Paxillin (60% per h; $P = 0.019$) up to 60 min after biopsy. The amount of total Paxillin decreased (28% per h; $P = 0.034$) over the same time course. In early bladder cancer, no changes were noted in any endpoints up to 45 min. Standardisation of the time taken between biopsy and fixation may be critical, particularly in studies using phosphorylated protein biomarkers.

Keywords Src-family kinases · Protein–tyrosine kinase · Pharmacology · Biopsy · Immunohistochemistry

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Introduction

A number of new anti-cancer drugs which target kinase signalling pathways have met with clinical success in recent years [1–3]. Many others are currently in pre-clinical and clinical development. As these drugs are hypothesised to work by inhibiting specific and pre-defined targets, the ability to demonstrate inhibition of the target in the desired disease tissue when administered to patients at tolerable doses could provide valuable data to support and guide continuing drug development. In many cases, such dynamic biomarker studies are likely to require samples of tumour tissue from patients receiving the drug, ideally comparing samples taken during treatment with those taken before treatment.

Kinase signalling is a dynamic process, and activity readings may be dependent on tissue handling, temperature, and oxygenation. Depending on the lability of the quantitatively assessed endpoint, even short delays before fixation or freezing of tissue may affect the levels of activity within specific kinase pathways. Failure to address the impact of such a delay on a specific biomarker may introduce additional variability and/or artefact into the results of pharmacodynamic studies. It is conceivable that a systematic difference in this delay between pre-treatment and on-treatment biopsies could lead to a false conclusion being drawn from the study. For example, where the pre-treatment sample is taken at the time of diagnostic

biopsy and the on-treatment sample is taken following excision of the tumour at the time of definitive surgery, there may be an extended period of time between devascularisation of the tumour and fixation of the post-excision sample.

Src kinase inhibitors are currently in early clinical development for the treatment of solid malignancies [4, 5]. This work was carried out preliminary to phase I trials of the novel Src kinase inhibitor AZD0530 (AstraZeneca, Alderley Park, UK). Pre-clinical studies have indicated several key phosphorylation events thought to be mediated, either directly or indirectly, by Src [6]. In this study we chose to investigate the levels of tyrosine phosphorylation in general as well as the phosphorylation of two signalling proteins known to be downstream of Src as potential dynamic biomarkers that could be used to demonstrate the pharmacodynamic effects of a Src kinase inhibitor. Paxillin and focal adhesion kinase (FAK) are both components of the focal adhesion complex, which are regulated by Src kinase during the process of cancer cell invasion. Src autophosphorylation (at Tyr 418) was not examined as preclinical data suggest that this is not a reliable marker of drug activity for AZD0530 (unpublished data). The reasons for this are currently under investigation.

The objective of the work presented here was to establish the impact of delay between biopsy and fixation on the measured activity of Src kinase in two different tumour types. The primary measures of Src kinase activity were the levels of phosphorylation of FAK and Paxillin. In addition, the change in expression of active Src over time was assessed using a semi-quantitative technique.

Materials and methods

Patients

Breast cancer

A total of 20 women with locally advanced breast cancer amenable to core biopsy were recruited from a single centre during 2003–2005 (City Hospital, Nottingham, UK). Recent radiotherapy to the biopsy site or incomplete healing from previous surgery were exclusion criteria.

Bladder cancer

A total of five patients undergoing transurethral resection of transitional cell carcinoma of the bladder, in whom there was tissue excess to diagnostic requirements, were enrolled in a single centre during 2004 (Addenbrooke's hospital, Cambridge, UK).

All patients were aged 18 years or over and all gave full written informed consent to participate in the study which was approved by the independent ethics committee for each participating centre.

Tissue sampling

Breast cancer

Up to 4 core biopsies were taken with a 14-gauge biopsy needle. The first core was divided in 2, one half being immersed immediately in liquid nitrogen for luminex analysis and the other half being placed immediately in isopentane for IHC which had been pre-cooled in liquid nitrogen. The remaining samples were placed on damp blotting paper. At the appropriate time point a sample was divided into two, one half being immersed in liquid nitrogen and the other in liquid nitrogen–pre-cooled isopentane. Time points explored in breast cancer were 10, 20, 30, 45 and 60 min after the time of biopsy. Not all time points were sampled in all patients, the time points for any one individual having been assigned at random.

Bladder cancer

Immediately after removal of the excess tumour material from each patient's bladder, it was divided into 11 samples each weighing approximately 50 μg. One sample was immediately placed in liquid nitrogen, one sample was immediately immersed in 10% neutral buffered formalin and one sample was immediately placed in liquid nitrogen–pre-cooled isopentane. The remaining samples were placed in normal saline awaiting delayed fixation. One sample was frozen in liquid nitrogen and one in pre-cooled isopentane at 10, 20, 30 and 45 min after the time of the tumour resection.

All frozen samples were stored at –80°C. Formalin-fixed specimens were processed and blocked in paraffin within 24 h and stored at room temperature.

Luminex assay

Sample preparation and lysis

Samples were shipped to a central laboratory (Alderley Park, Macclesfield, UK) on dry ice. The frozen samples were mechanically disrupted together with a 15-fold (wt/vol) quantity of frozen lysis buffer, pH 7.4 [10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 1% Triton-X100, 10% Glycerol, 0.1% SDS, 0.5% Sodium Deoxycholate, 1 mM PMSF and 5% Sigma P2714 Protease Inhibitor

Cocktail (Sigma Aldrich Co. Ltd, Poole, Dorset, UK)] using a SPEX 6850 liquid nitrogen freezer mill (SPEX CertiPrep, Metuchen, NJ, USA) to produce a mixed powder of tissue and lysate solution at –80°C. Powdered samples were thawed and shaken at 4°C for 30 min prior to clarification by centrifugation (4°C, 20 min, 4,500 g, Heraeus Biofuge Fresco benchtop unit with a #3325 24 place rotor). The supernatants were retained and quantified for protein using the Pierce BCA optical protein assay (Pierce and Warriner, Chester, UK). Lysates were stored at –80°C prior to Luminex assay.

Assay standard

A mouse NIH 3T3 cell line transfected to over-express active Src kinase (c-Src3T3) was cultured, as previously described [7], to approximately 80% confluence, rinsed with phosphate buffered saline and lysed in situ using the lysis buffer described above. The lysate was vortexed and rotated at 4°C for 30 min before clarifying by centrifugation as above.

Luminex bead conjugation

Rabbit polyclonal capture antibodies were coupled to Luminex beads using the BioRad Amine coupling kit as described in the manufacturer's instructions (bead sets and kit from Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK Other reagents: Sigma Aldrich Co. Ltd, Poole, Dorset, UK). Antibodies were supplied without BSA or azide. Antibody (5 μg) per reaction was used for both anti-FAK pY861, and anti-Paxillin pY31 (Biosource International, Camarillo, CA, USA) and 10 μg total Paxillin antibody per reaction (Cell Signaling Technology, Inc., Beverly, MA, USA).

Luminex assay

All dilutions and washes were done with Tris Buffered Saline pH 7.4 + 1% BSA + 0.5% Tween 20 + 0.05% sodium azide (TBSBNT buffer). All assays were performed as single endpoint per well in Millipore MABVS1210 filter plates. (Millipore Corporation, Bedford, MA, USA). A Millipore 96-well MultiScreen Vacuum Manifold was used for the wash steps. Plates were prewetted/washed with 100 μl TBSBNT per well which was removed by a brief vacuum step. Twenty five micro liter of diluted bead antibody conjugate (at 100 beads/μl) plus 75 μl TBSBNT was added to each well and vacuumed followed by a further 100 μl TBSBNT wash and vacuum step. Lysate dilution (100 μl) was then added per well. A c-Src3T3 lysate standard dilution range was made starting at 100 μg/ml and the test lysates were diluted to 175 μg/ml. All samples and standards were run in triplicate. The plates were sealed and incubated for 16 h in the dark on a plate shaker set at 300 rpm following a 30 s 1,100 rpm mix. The plates were washed 3 times and 50 μl/well Phycolink® anti-Phosphotyrosine(type 1)R-Phycoerythrin conjugate (Prozyme via Europa Bioproducts Ltd, Ely, Cambs., UK) added prior to incubation whilst shaking in the dark for a further 1 h before a 100 μl/well wash, a final addition of 120 μl/well TBSBNT and being read on the Bio-Rad BioPlex using the BioPlex Manager v3 for data collection and quantitation of the unknowns against the c-Src3T3 standard curves.

In the total Paxillin assay, the c-Src3T3 lysate concentration was 300 μg/ml and the test lysate concentration was reduced to 100 μg/ml. Detection for these plates was by 1 h incubation with 50 μl/well anti-Paxillin (BD Transduction Laboratories, Franklin Lakes, NJ, USA) (diluted 1 in 83) followed by 1 h with 50 μl/well diluted (1 in 100) anti-mouse IgG/biotin [Upstate Biotechnology (UBI), Lake Placid, NY, USA] and a further 1 h with 50 μl/well (diluted at 1 in 100) Streptavidin–Phycoerythrin conjugate (UBI) with three wash steps between each stage. The beads used for the total phosphorylation assay were Qiagen LiquiChip Avidin Beads (Qiagen GmbH, Hilden, Germany) which were allowed to bind 75 μl/well of a 1:1,600 dilution of Cell Signalling Technology pY100-Biotin prior to washing and overnight lysate capture.

Although it would have been desirable to measure Src and total FAK expression using this technique, appropriate reagents could not be identified to provide robust assays using this platform (data not shown).

Immunohistochemistry

Samples for frozen-tissue IHC were mounted on cork discs prior to immersion in isopentane which had been precooled in liquid nitrogen for 15 min. After immersion in chilled isopentane, samples were placed in freezing tubes (Nalgene Labware, Hereford, UK) and placed into liquid nitrogen. Samples were shipped to the central laboratory on dry ice. For formalin-fixed tissue, the samples were left in 10% neutral buffered saline for 24 h. They were then processed and embedded in paraffin wax. Samples were shipped to the central laboratory at room temperature.

For frozen samples, 7 μm thick sections were cut using a cryostat (model CM3050S, Leica Microsystems UK Ltd, Milton Keynes, UK) and mounted on glass slides. For formalin-fixed, paraffin-embedded samples, 4 μm thick sections were cut and mounted on glass slides.

All sections were processed using the Dako Envision Detection Kit-HRP Mouse according to the manufacturers

instructions (DakoCytomation, Cambs., UK). The primary antibody was Mouse Monoclonal Clone 28 (supplied by Dr Hisaaki Kawakatsu, Dean Shepard Lab, San Francisco, CA, USA. Stock concentration 1.9 mg/ml, assay concentration 1 μg/ml), which targets the C-terminal tail of Src, which is only detectable when Src is in its active conformation [8].

All samples were scored according to the intensity of staining of carcinoma cells on a scale of –, ±, +, ++, +++ by one observer (VJ) and confirmed by an independent reviewer.

IHC for FAK, phospho-FAK, Paxillin and phospho-Paxillin was not carried out in this study as these assays produce poor quality data using frozen tissue (data not shown).

Statistical methods

Previous experience had indicated that the luminex data would conform to a log normal distribution, therefore the data were log transformed prior to analysis. Missing values and zero values were excluded from the analysis as these samples were assumed to contain no tumour cells. Trends over time were formally explored by fitting the following model to each variable:

$$E = E_0 + a \times \text{time},$$

where E is the endpoint, E_0 is the value of the intercept, time is the time the sample was frozen and a the model parameter to be determined. The robustness of these assumptions was checked by examining the residuals and also by performing the analysis on non-transformed data.

The model was fitted to each of the endpoints using the SAS procedure 'proc mixed'. Time was fitted as a fixed effect and patient was fitted as a random effect.

For this analysis in patients with breast cancer the actual recorded time of freezing was used.

Literature sample search

All papers from the Medline Core Clinical Journals [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi (accessed 16 October 2006)] collection added between January 2000 and February 2006 were searched using the term biopsy AND cancer AND (biomarker OR marker) AND (dynamic OR phospho* OR kinase OR pharmacodynamic). The sample was restricted to English language journals publishing abstracts on Medline. From this sample, all reports of original research containing data derived from biopsies of clinical tissue where assays of dynamic enzyme activity endpoints were reported were included in the survey.

Results

Patients

A total of 20 women with locally advanced breast cancer and 5 patients undergoing transurethral resection of large transitional cell bladder tumours were enrolled into the study (Table 1). All patients underwent biopsies.

Histological and IHC evaluation

The methodology did not permit independent histological validation of each specimen used in the Luminex analysis. To estimate the proportion of biopsies that may or may not have contained evaluable tumour tissue, a frozen sample was taken at each time-point for histological analysis. Furthermore, as the study was designed to assess likely biomarkers of Src kinase activity, these samples were assessed for expression of active Src kinase. In addition, baseline paraffin embedded tissue (PET) was collected from the bladder tumours. All samples that contained tumour tissue expressed active Src kinase (as demonstrated by the clone 28 antibody) staining all tumour cells uniformly. All immediate fixation samples were graded either ++ or +++ in intensity of staining using this assay. There was no trend to a change in intensity of clone 28 staining over time in either tumour type (data not shown). All bladder tumour samples, but not all breast tumour biopsies contained tumour cells (Table 2).

Inter-patient variability

For each luminex variable the inter-patient variability of the logarithmically transformed data at time zero was calculated. The results are given in Table 3.

Effects of delayed freezing on biomarker results

Figure 1 shows the effects of delayed freezing on the levels of each marker in each tumour type. In breast cancer there

Table 1 Patient characteristics

	Breast (N = 20)	Bladder (N = 5)
Female/male	20/0	2/3
Mean age (range)	67.3 (47–82)	71 (65–83)
Prior breast surgery	4	NA
Prior TURBT	Na	0
Prior chemotherapy	5	0
Prior hormone/immunotherapy	6	0
Prior radiotherapy	3	0

NA not applicable

Table 2 Histological validation

	Number of samples (%)		
	Breast	Bladder	
		Isopen	PET
Total	60	24	5
No. of tumour cells	11 (18)	0	0
Very few tumour cells (<10%)	4 (7)	0	0
Uninterpretable[a]	17 (28)	0	0
Analyzable for active Src expression	32 (53)	24 (100)	5 (100)

Proportion of biopsies which contained tumour (assessed by histochemistry)

[a] Uninterpretable due to damage during the antigen retrieval process or not possible to cut sections

Table 3 Inter-patient variability of luminex data in breast cancer

Endpoint	Inter-patient variance (SD)	*N*
Paxillin31/total Pax	0.1512 (0.3888)	19
Paxillin 31	0.6071 (0.7791)	19
FAK861	0.4090 (0.6396)	15
Total phosphotyrosine	0.9677 (0.9837)	10
Total Paxillin	0.3701 (0.6084)	20

Zero values were excluded from this analysis. Data are for the logarithmically transformed values expressed in cSrc3T3 lysate equivalent μg/ml

N number of samples included in each analysis

was a statistically significant increase in levels of phosphorylated Paxillin over time (where $P = 0.019$, based on the effect of time in the statistical model), whilst there was a decrease in the total level of Paxillin ($P = 0.034$). This resulted in a significant increase in the ratio of phosphorylated Paxillin to total Paxillin ($P < 0.0001$). The changes in levels of total phosphotyrosine over time, though statistically significant ($P = 0.098$), were heavily influenced by the outlying result at 60 min. This effect was, therefore, considered insignificant. There was no significant trend detected in levels of phosphorylated FAK. In early bladder cancer, there was no detectable, statistically significant change in the levels of any of these markers with delayed freezing.

The likely effect of delayed fixation in advanced breast cancer was modelled statistically. The fitted effects for percent change from baseline are illustrated in Fig. 2.

Literature sample

A survey of recently published studies examining dynamic endpoints in clinical tumour samples revealed that little attention has been paid to reporting the time between biopsy and sample fixation. In particular, studies employing IHC techniques rarely standardised or minimised this variable. These data are summarised in Table 4.

Discussion

Quantitative pharmacodynamic biomarkers of kinase activity are an attractive tool to guide early clinical development of novel kinase inhibitors. To date, only a few studies have been published using such biomarkers derived from human tissue [47, 48].

IHC assessments of markers of proliferation, such as Ki67, and apoptosis, such as cleaved caspase 3, have been used in drug studies with some success [49, 50]. Some studies have addressed the issue of delayed fixation on these endpoints in human tissues: One study examined the effect of delays in formalin fixation of up to 24 h for a variety of human tumours and concluded that there was no significant decrease in mitotic activity [51]. In another study, the proliferating cell nuclear antigen index (PCNA) (determined by IHC) increased by 10% when fixation was delayed by 24 h in breast cancer specimens [52]. A third report demonstrated a 53% increase in the number of mitotic figures in human breast samples where fixation was delayed for 6 h [53]. A similar effect was seen in normal colonic mucosa [54]. In a preclinical model of sarcoma, a more dramatic effect was seen with a 50% decline in mitotic index over 1 h [55]. Despite these conflicting data, none of the studies of proliferation markers included in the literature survey presented here accurately recorded the true time from biopsy to fixation.

There may be a number of factors contributing to any change in signal that arises during delayed fixation. As kinase activity is dependent upon the intracellular supply of ATP, we might predict that hypoxia would be a dominant factor. Pre-clinical models of cerebrovascular injury suggest increased activity of the mitogen activated kinase pathways in the hours following stroke [56, 57]. Clearly, this may be part of the cell's defensive action against hypoxia, and the effects seen in the current study may also reflect such defensive activity (although this is not a recognised activity of Paxillin).

The data presented here are in keeping with an increased activity of Src kinase family members in locally advanced breast cancer during delayed fixation. Focal adhesions may be targeted for degradation following phosphorylation by Src [58], accounting for the apparent loss of total Paxillin and increase in levels of phosphorylation over time. Appropriate reagents to measure total FAK concentration using Luminex are not currently available, so it is possible that the observed lack of a concomitant increase in FAK

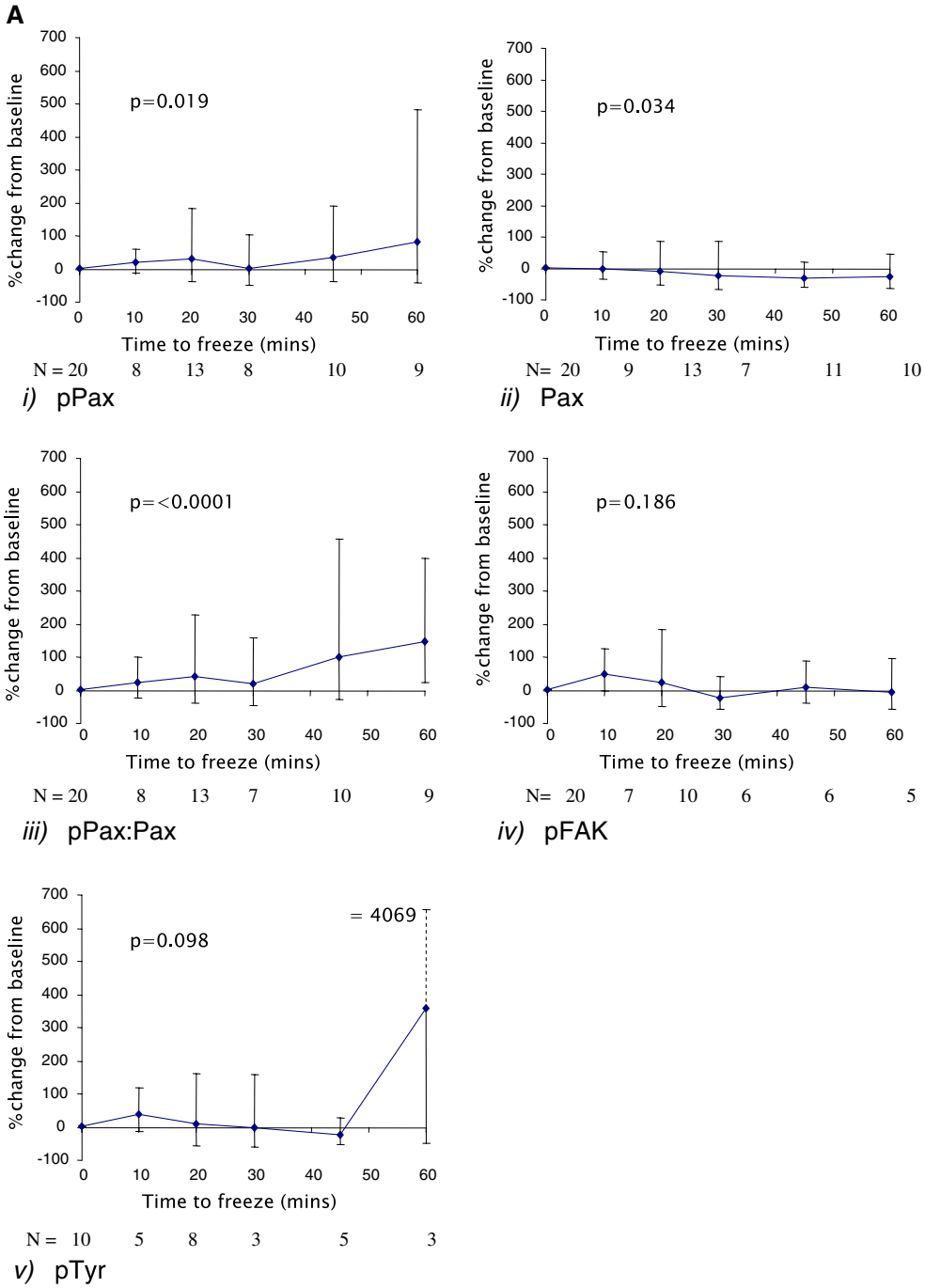

Fig. 1 Changes in levels of phosphorylation-dependent endpoints over time as measured by luminex. **a** Locally advanced breast cancer. **b** Early bladder cancer. (i) pPax (ii) Pax (iii) pPax:Pax ratio (iv) pFAK (v) pTyr. Values are the geometric mean percentage change from baseline. *Error bars* are one standard deviation (calculated from log transformed data then exponentiated)

phosphorylation is due to a loss of total FAK. Total phospho-tyrosine is a less specific measure, and the observed changes here may be due to increased activity of a variety of different kinases. The failure to demonstrate increased levels of active Src expression by IHC reflects the high readings in this assay at baseline and the semi-quantitative nature of the assay. Thus it is not likely that the assay technique could detect a significant rise in activity.

The lack of effect in early bladder cancer is intriguing. This may simply reflect the small number of patients being insufficient to demonstrate a change in biomarker activity

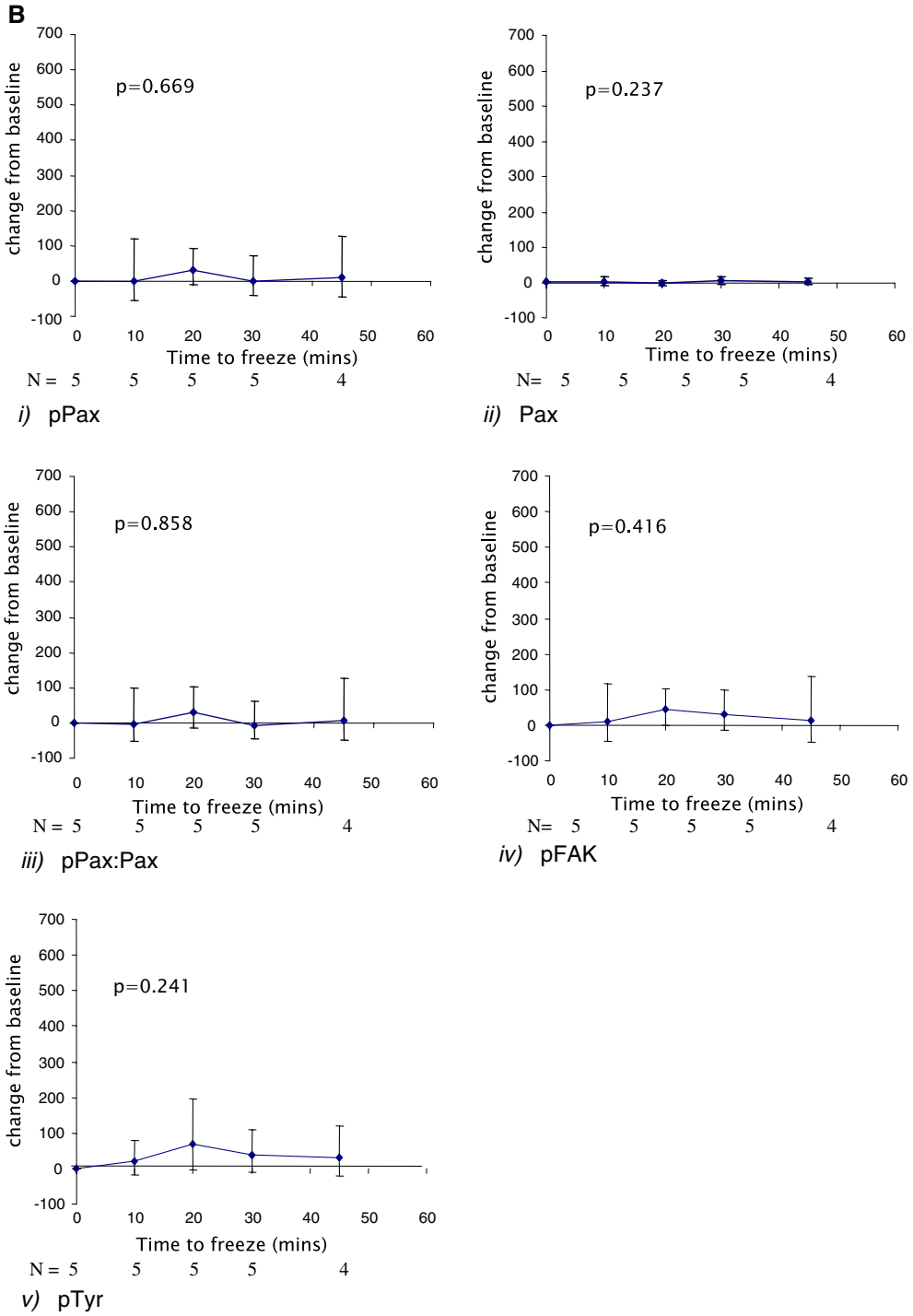

Fig. 1 continued

over time. Alternatively, this may reflect different biology within early bladder tumours compared with more advanced tumours of the breast.

One concern with the Luminex assay used here is that it does not offer the opportunity to control for intra-tumoural tissue heterogeneity, which we have previously shown to cause significant variability in readings between different samples both at the intra-tumoural and inter-patient level [59]. Intra-tumoural heterogeneity is not likely to explain the change in signal over time in the breast samples as we would predict that any effect due to heterogeneity would be entirely random rather than time-dependent.

This study demonstrates that, in locally advanced breast cancer, delayed fixation causes a statistically significant

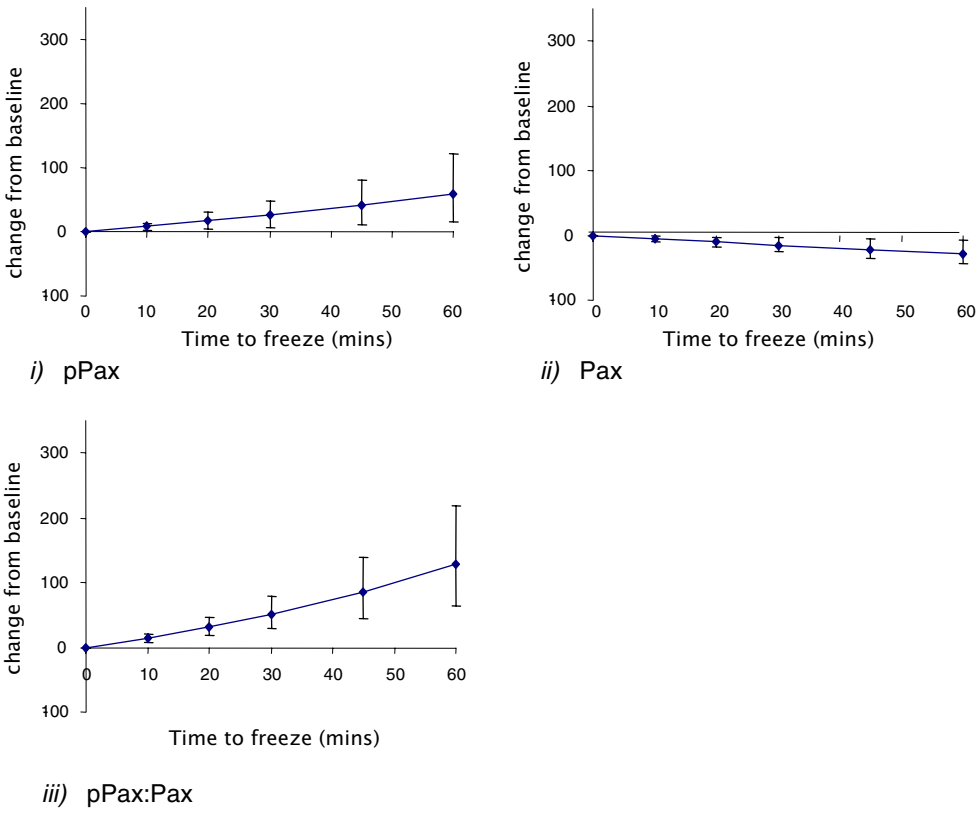

Fig. 2 Changes in levels of phosphorylation-dependent endpoints over time in locally advanced breast cancer as fitted by the statistical model. Statistical modelling of the data displayed in Fig. 1a suggests a statistically significant effect of delayed time to freeze on (i) pPax (ii) Pax (iii) pPax:Pax ratio. *Error bars* are 90% confidence intervals according to the model

Table 4 Survey of recent publications reporting enzyme activity assays in clinical tumour specimens

Primary activity assay	Nature of assay	*N*	Details of time to fixation	References
Src kinase	Immune complex kinase assay	2	Immediately after resection (both studies)	[9, 10]
pErk; pAkt; p38; pRB	Phospho-specific IHC	7	No details given	[11–17]
Cell cycle (Ki-67/ PCNA)	IHC	19	No details given.	[17–36]
Thymidine kinase	Lysate-based activity assay	2	Immediately after tumour confirmed on frozen section (1 study); not stated (1 study)	[37, 38]
Telomerase	TRAP	8	No detail (1 study); others range from 'immediate' to 'within 60 min'	[39–46]

Publications were sampled by using medline search criteria. All studies were published since 2000

PCNA proliferating cell nuclear antigen; *IHC* immunohistochemistry; *TRAP* telomeric repeat amplification protocol

increase in kinase signalling during 60 min after biopsy from the oxygenated tumour site. Intriguingly, this effect is not observed in early bladder cancer, although this may simply be due to the small number of patients within this group.

These findings emphasise the importance of standardising the time between biopsy and fixation in pharmacodynamic studies examining these endpoints. In addition, this effect may need to be established for different tumour types and different dynamic endpoints. In particular, this raises significant concerns when comparing biopsies taken from oxygenated tissue with biopsies taken from tumours that have become hypoxic for significant periods of time (e.g. due to vascular ligation prior to complete resection during surgery) prior to sample collection.

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