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Oestrogen-regulated Genes in Breast Cancer: Association of pLIV1 With Lymph Node Involvement

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In order to isolate markers of oestrogen responsiveness in breast cancer, we have cloned a number of oestrogen-regulated genes. Two of these, pLIV1 and pLIV2 (pS2), have been shown to be predominantly expressed in oestrogen receptor (ER)+ tumours. In this study, we examined their expression in relation to various clinical and histopathological features of breast cancer, and showed that pLIV1, but not pS2, is significantly associated with lymph node involvement ($P<0.01$), while pS2 is more frequently observed in premenopausal patients ($P<0.05$). Subdivision of the pLIV1 data by ER and nodal status of the tumour identified a highly significant association between pLIV1 expression and lymph node involvement in ER-positive disease, with 15/24 (63%) ER+ pLIV1+ tumours showing nodal involvement. Conversely, 20/23 (87%) ER+ pLIV1− patients were lymph node-negative ($P<0.001$). Subdivision of the pS2 data by ER status did not reach significance. The application of pLIV1 as a marker of lymph node involvement was further exemplified in small tumours (≤ 2 cm), where 11/12 (92%) lymph node-positive patients expressed pLIV1, while 17/22 (77%) node-negative patients were pLIV1 negative ($P<0.001$). Similarly, pLIV1 expression identified lymph node involvement in moderately differentiated tumours ($P<0.01$), but was independent of vascular invasion. pLIV1 may, therefore, represent a candidate gene for metastatic spread in ER+ breast cancer.

Key words: breast cancer, oestrogen-regulated genes, oestrogen markers, lymph node involvement
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INTRODUCTION

THE GROWTH of breast cancers is often oestrogen responsive and the oestrogen receptor (ER) has provided a useful marker in the management of the disease. However, with only 65% of ER+ breast cancers responding to endocrine therapies, the need for other markers of responsiveness is apparent [1].

The measurement of a final product of oestrogen action may provide such a marker, since it would identify those patients with a functional receptor system. In this light, we have isolated 10 oestrogen-inducible genes (pLIV1, pLIV2 and pSYD1-8) and one gene (named pMGT1) whose level of expression is reduced by oestrogen but increased by anti-oestrogens [2–4]. We have shown previously that two of these genes, pLIV1 and pLIV2, identical to the pS2 gene [5], are significantly associated with ER+ disease ($P<0.001$), and as such, may indicate ER functionality. Interestingly, however, and despite their oestrogen inducibility, pLIV1 and pS2 were not always co-expressed in ER+ breast cancers, an observation that suggested additional and gene-specific regulatory elements which may relate to the different fuctional roles of these gene products [6]. This concept is supported by the current study where we examined the expression of pLIV1 and pS2 in relation to various clinical and histopathological features of breast cancer, and have shown that pLIV1, but not pS2, is significantly associated with lymph node involvement, while pS2 is more frequently observed in premenopausal patients.

MATERIALS AND METHODS

Tumour samples were obtained from 74 patients presenting during 1990 with primary breast cancer at the City Hospital in Nottingham under the care of Professor R.W. Blamey. A simple or subcutaneous mastectomy was undertaken, and lymph node biopsy samples removed from the lower axilla, from the apex of the axilla, and from the internal mammary chain. Patients with tumour cells histologically evident in any node were classified as lymph node positive. The menopausal status and age at mastectomy of each patient were recorded in addition to tumour size. Histological grade of the malignancy was assessed in all tumours using a modification of Bloom and Richardsons criteria [7]. Tumours were graded I to III with increasing loss of

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differentiation. The mitotic activity of tumours was assessed by counting the number of mitotic figures in 10 or 20 high power fields at the peripheral infiltrating margin of the tumour. Grades I–III corresponded to 0–9, 10–19 and over 20 mitotic figures/10 high power fields, respectively.

Tissue analysis

Immediately after surgery, the tissue was snap-frozen and stored in liquid nitrogen before transportation in dry ice to the Tenovus Centre. Samples were stored at −70°C until assay.

The assay procedures for the measurement of ER by immunohistochemical analysis using the ER rat monoclonal antibody H222spγ [8], and pLIV1 and pS2 by northern analysis have been described in detail elsewhere [6]. Briefly, the specimen evaluation for ER was performed on an Olympus microscope (BH-2) using ocular magnification of x 40. Control slides (control rat IgG antibody) were checked for non-specific binding. Tumours were classified as ER+ where > 5% of tumour cells were stained for the receptor [9]. Northern analysis of RNA from each tumour and densitometric assessment of the 4.4 kb pLIV1 and 0.6 kb pS2 mRNA transcript were performed, and a cut-off value (to exclude background hybridisation) assigned as described previously [10].

Statistical analysis

Contingency tables (χ^2) with Yates correction factor were used to compare subgroups of the tumour population.

RESULTS

The data presented in Table 1 shows the clinical (lymph node status, menopausal status and tumour size), pathological (histological grade and vascular invasion) and biochemical (ER status) features of the primary tumours used in the current study. The distribution of these parameters is similar to previous reports in the Tenovus/Nottingham primary breast cancer series [6, 9, 10].

Examination of the relationship between the clinical and pathological data (Table 2) revealed significant associations between pLIV1 and lymph node involvement ($P<0.01$) and pS2 and menopausal status ($P<0.01$). Thus, while 16/29 (55%) lymph node-positive patients were pLIV1-positive only, 10/45 (22%) lymph node-negative patients showed evidence for expression of this gene sequence. No other relationships were significant.

As described previously [6], the pLIV1 and pS2 genes were most frequently detected in ER+ disease (where pLIV1 was expressed in 51% of ER+ tumours compared to only 7% of ER− tumours, and pS2 was similarly observed in 47% of ER+ tumours compared to 7% of ER− tumours). Consequently, the expression of these genes was further examined in the ER+ subset of patients (Table 3). Significantly, the relationship between pLIV1 and lymph node involvement was strengthened ($P<0.001$) by the exclusion of the ER− tumours. A total of 15/24 (63%) ER+pLIV1+ tumours showed nodal involvement while 20/23 (87%) ER+pLIV1 − tumours were lymph node-negative. The association of pS2 and menopausal status did not

Table 1. Patient and tumour characteristics

	No. of patients	Percentage
Menopausal status		
Pre-	31	42
Post-	43	58
ER status		
ER+	47	63
ER−	27	27
Nodal status		
Node-negative	45	61
Node-positive	29	39
Grade		
I	16	22
II	25	34
III	33	44
Size		
⩽2 cm	53	72
>2 cm	21	28
Vascular invasion		
Negative	51	69
Positive	23	31

Table 2. Relationship between oestrogen-regulated gene expression and clinical and pathological features

	pLIV1+	pLIV1−	*P* value	pS2+	pS2−	*P* value
Menopausal status						
Pre-	13	18	n.s.	14	17	<0.01
Post-	13	30		10	33	
Stage						
Node-negative	10	35	<0.01	12	33	n.s.
Node-positive	16	13		12	17	
Grade						
I	4	12		4	12	
II	12	13	n.s.	9	16	n.s.
III	10	23		11	22	
Size						
⩽2 cm	17	36	n.s.	20	34	n.s.
>2 cm	9	12		4	16	
Vascular invasion						
Negative	15	36	n.s.	13	38	n.s.
Positive	11	12		11	12	

n.s., non-significant.

Table 3. Relationship between oestrogen-regulated gene expression and clinical and pathological features in ER+ disease

	ER+pLIV1+	ER+pLIV1−	*P* value	ER+pS2+	ER+pS2−	*P* value
Menpausal status						
Pre-	12	10	n.s.	13	9	=0.06
Post-	12	13		9	16	
Stage						
Node-negative	9	20	<0.001	12	17	n.s
Node-positive	15	3		10	8	
Vascular invasion						
Negative	14	19	n.s.	13	20	n.s.
Positive	10	4		9	5	

n.s., non-significant.

reach significance in ER+ disease, while insufficient numbers (2/27) precluded further analysis of pLIV1 and pS2 expression in ER− disease (data not included). In addition, no significant relationship was observed between pLIV1 or pS2 and vascular invasion.

Subdivision of the data shown in Table 3 by the pLIV1-independent variable of tumour size showed pLIV1 expression to be highly predictive of lymph node involvement in small cancers (≤ 2.0 cm): 92% patients with lymph node involvement were pLIV1 positive, while only 23% lymph node-negative patients were pLIV1-positive (Table 4). pLIV1 expression also identified lymph node involvement in moderately differentiated cancers, with well differentiated tumours being predominantly pLIV1-negative ($P=0.01$). In addition, the relationship between pLIV1 and nodal involvement was similar in the presence or absence of vascular invasion. Similarly, stratification of the pLIV1 data by pS2, showed that the association of pLIV1 and lymph node status was independent of pS2, expression in ER+ tumours (data not shown).

DISCUSSION

We have described previously the cloning of a number of oestrogen-responsive genes from the T-47D and ZR-75-1 human breast cancer cell lines [2–4]. Interestingly, two sequences, pLIV1 and pS2 (pLIV2), which are both regulated by oestrogen in culture, are not always co-expressed in the same clinical specimen [6]. Although the reasons for this are currently unclear, it is likely to be determined by gene-specific differences in promoter regions, such that one gene may fail to discriminate between a normal or mutant ER, while another may be over-ridden by a responsiveness to additional regulatory factors. It is this diffential expression which may contribute to the biological behaviour of breast cancer, a concept which is supported by the present study, where a differential relationship with a number of clinical and pathological features of breast cancer has been observed. Thus, while pLIV1 is highly and significantly related to lymph node involvement in ER+ tumours, pS2 expression is observed more frequently in ER+ premenopausal disease. This latter observation is similar to those previously reported [11, 12] where a predominance in expression in tumours from premenopausal females [13] and a lack of association with nodal involvement [14] have been observed.

Table 4. Relationship between pLIV1 expression and clinical and pathological features in ER+ disease

	ER+pLIV1−	ER+pLIV1+	*P* value
Tumour size (≤2 cm)			
N−	17	5	<0.001
N+	1	11	
Tumour size (>2 cm)			
N−	3	3	n.s.
N+	2	5	
Grade I			
N−	8	2	n.s.
N+	1	1	
Grade II			
N−	6	4	<0.01
N+	0	8	
Grade III			
N−	6	3	=0.1
N+	2	6	

N−, node negative; N+, node positive; n.s. non-significant.

Of greatest interest, however, was our observation showing a highly significant association between the presence of pLIV1 in primary breast cancers and lymph node involvement. Since nodal involvement still remains the single best predictor of recurrence [15], pLIV1 expression may be extremely useful in identifying those tumours with apparent similar phenotypes (i.e. ER positivity) that display differing metastatic potential.

While sequence analysis of the pLIV1 clone (which encodes approximately 50% of the full length sequence) has failed to reveal any significant homologies, the predicted amino acid sequence has exposed an imperfect zinc finger motif (personal communication, Gullick and Bates, ICRF, London, U.K.). Its presence, however, in 92% of smaller ER+ primary tumours (≤ 2 cm in diameter) with positive lymph nodes raises interesting possibilities, particularly with regard to breast cancer screening, where the first 5 years of screening have revealed smaller tumours in screen-detected compared to their symptomatic counterparts. It is possible, therefore, that pLIV1 expression in these tumours will provide a useful marker of lymph node involvement and of the need for axillary surgery.

In summary, the data presented in the current paper expand our observations that pLIV1 and pS2 are preferentially and variably expressed in ER+ tumours, and show that differences in their expression may be of significance in defining their biological role (and, therefore, more accurately defining a prognostic use) in the clinical management of breast cancer.

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Suramin Inhibits Growth and Transforming Growth Factor-β1 (TGF-β1) Binding in Osteosarcoma Cell Lines

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Autocrine production of growth factors has been shown to be involved in the multistep process of tumorigenesis. The ability of suramin, a polyanionic anti-parasitic drug, to block growth factor-induced cell proliferation makes it a potential antineoplastic drug. We studied the effects of suramin on seven osteosarcoma cell lines. Using clinically achievable concentrations of suramin (50–400 μg/ml), we found a time- and dose-dependent inhibition of [^{3}H]thymidine incorporation. We also showed that suramin is able, dose-dependently, to prevent binding of transforming growth factor (TGF)-β1 to its receptors. DNA synthesis inhibition by suramin was attenuated by TGF-β1 in some cell lines. Two cell lines that were inhibited by TGF-β1 were affected similarly by suramin as cell lines that were stimulated by TGF-β1. In conclusion, in five out of seven osteosarcoma cell lines, we showed a correlation between inhibition of growth factor-stimulated mitogenesis and binding of TGF-β1 to its receptor. Similar effects in TGF-β1-inhibited osteosarcoma cell lines suggest involvement of other mechanisms and/or growth factors. However, suramin proves to be a potent inhibitor of osteosarcoma cell proliferation *in vitro*.

Key words: suramin, transforming growth factor-β1, osteosarcoma, receptor, autocrine growth
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INTRODUCTION

THE GROWTH of normal cells and tissues is a delicately balanced system in which a key role is played by multiple polypeptide growth factors. One of these factors is transforming growth factor-β (TGF-β) a 25-kDa homodimer, originally described as being able to induce phenotypic transformation of normal rat fibroblasts [1–3]. Subsequently, TGF-β has been shown to be multifunctional, and to be expressed ubiquituously, being important for cell growth and differentiation as well as wound healing. In contrast to its well characterised inhibitory effects on epithelial derived cells, TGF-β stimulates proliferation of mesenchymal cells [1–4]. Although the pathways of TGF-β are