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The Expression of Milk Fat Globule Antigens Within Human Mammary Tumours: Relationship to Steroid Hormone Receptors and Response to Endocrine Treatment

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Abstract—*The value of steroid hormone receptors for the management of advanced carcinoma of the breast is often limited by the lack of availability of fresh tissue. Differentiation antigens may be estimated on paraffin-embedded fixed material by immunostaining, and the aim of this study was to determine whether staining with the monoclonal antibody raised to human milk fat globule (HMFG-1) could replace receptor measurements.*

The indirect immunoperoxidase technique was used to stain formalin-fixed paraffin-embedded tumour samples from 168 patients. All received tamoxifen or ovarian ablation as first-line systemic therapy, and all were evaluable for response (UICC criteria). One hundred and sixty-seven had oestrogen (ER) and progesterone receptors (PR) estimated. HMFG-1 staining was assessed as the percentage of tumour cells stained, and by the site of stain. The proportion of cells stained was highly correlated with both ER ($P < 0.0001$) and PR ($P < 0.0001$) and with response. When ≥30% cells stained, 53 of 69 (77%) responded; when 20–29% stained 10 of 19 (53%) responded, when 10–19% stained seven of 19 (37%) responded, and when ≤9% cells stained 16 of 61 (26%) responded ($P < 0.0001$). The median survival of patients with tumours that stained ≥30% cells was 36 months, and with no cells stained, 11 months ($P < 0.0001$). ROC (receiver operator characteristic) curves found that the optimum threshold for sensitivity and specificity of response prediction was ≥20% cells stained. Cox's multiple regression analysis of 42 variables indicated that PR was the most important predictor of survival ($P < 0.000001$), but that after PR the percentage of cells stained with HMFG-1 was the most important ($P < 0.0001$). We conclude that immunostaining for HMFG-1 gives similar information to receptor status, and has the advantage that fixed archival tissue may be used.

INTRODUCTION

HUMAN MAMMARY TUMOURS are highly heterogeneous and the expression of a variety of proteins, such as oestrogen receptor, carcinoembryonic antigen and milk fat globule antigens, is usually variable within single tumours. Endocrine treatment for advanced cancer of the breast is valuable for palliation, but despite some clinically complete remissions, is never curative. This suggests that tumour stem cells may be resistant to treatment, but that, in a proportion of tumours, their progeny are responsive. Histologically differentiated tumours are more responsive than anaplastic ones [1]. We have investigated further the hypothesis that response might depend upon the degree of tumour differentiation. The aim of this study was to investigate the relationship between the expression of a putative differentiation antigen found on the human milk fat globule membrane (HMFG-1), and steroid hormone receptor proteins and response to endocrine treatment. The possible value of HMFG-1 as a potential marker for hormone responsiveness was investigated.

The monoclonal antibody HMFG-1 identifies antigens found on the milk fat globule membrane, and present on the surface of normal mammary epithelium and within ducts. It binds to complex mucin-like moieties of high molecular weight (≥300 K), as well as to smaller glycoproteins [2, 3]. These mucin-like molecules are found in malignant epithelial cells, on the cell membrane, in the cyto-

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plasm, and sometimes extracellularly. The pattern and intensity of staining varies greatly between one tumour and another, and sometimes within different areas of the same tumour [4, 5]. This makes objective assessment difficult. Various methods of assessment including scoring systems have been used, but results are conflicting [3–8]. To date the numbers studied are small, and there is an obvious need for further work on larger numbers of patients.

PATIENTS AND METHODS

Patients

The tumours of 168 patients with advanced breast cancer were studied. All were treated with endocrine therapy as first systemic treatment between 1975 and 1983, at the University Hospital of South Manchester. All were evaluable for response to endocrine manipulation according to internationally accepted criteria [9], and had had tumour steroid hormone receptors measured.

Response was assessed as complete (CR) when all measureable disease disappeared, partial (PR) when the size of measureable lesions decreased ≥50% in at least two planes, and static (SD) when the disease remained unchanged for a minimum of 6 months. Non-responding tumours were categorized as progressive disease (PD).

Treatment was ovarian ablation for premenopausal and tamoxifen for postmenopausal patients. Patients were reviewed at 1–2 month intervals: their clinical details are listed in Table 1. Karnofsky performance (KP) status was assessed for every patient at each clinic visit.

Table 1. Clinical characteristics of patients

Number	$n = 168$
Age	
mean = 60 years	
median = 60 years	
range 25–90 years	
Menopausal status	
Premenopausal	19 (12%)
Postmenopausal	149 (88%)
T stage	
T1	13 (7%)
T2	77 (45%)
T3	27 (16%)
T4	51 (30%)
Site of disease	
Soft tissue	127 (74%)
Bone	69 (40%)
Lung and pleura	50 (29%)
Liver	5 (3%)
Disease status	
Advanced primary	51 (30%)
Recurrence	117 (70%)
Treatment	
Tamoxifen	149 (88%)
Ovarian ablation	19 (12%)
Response to endocrine treatment	
Complete response	20 (12%)
Partial response	33 (20%)
Static disease	35 (21%)
Progressive disease	80 (47%)

Histopathology

Sections of all tumours were processed routinely by a single pathology laboratory. Tissue was fixed for up to 48 h in buffered formalin, and subsequently embedded in paraplast medium on a Shandon tissue processor. Representative blocks were sectioned at 4 μm thickness and reviewed in a coded and randomized order by a single experienced pathologist (L.T.). The type of tumour, presence or absence of elastosis and invasion of blood vessels or lymphatics were recorded. Infiltrating duct carcinomas were graded [10].

Receptor assays

Tissue was stored in liquid nitrogen, then homogenized by means of a pre-cooled Teflon capsule and tungsten ball, subjected to the action of a dismembranator for 30 s. The resulting powder was suspended in buffer (10 mM Tris–HCl pH 7.4 with 1 mM EDTA, 0.5 mM dithiothreitol and 30% v/v glycerol), and centrifuged at 100 ***g*** for 10 min at 4°C to remove nuclei, fat, and any debris. The dextran-coated charcoal assay was used to measure ER and PR: values ≥5 fM/mg cytosol protein were taken as positive [11].

Immunostaining technique for HMFG-1

HMFG-1 was raised in BALB/c mice as described by Taylor-Papadimitriou and colleagues, and was a gift from them [12]. HMFG-1 was applied as tissue culture supernatant diluted 1:10 in Tris-buffered saline which contained 20% normal rabbit serum. From tumours of 168 patients 310 separate blocks were examined. The paraffin sections were dried onto plain glass slides at 60°C, then dewaxed in either xylene (5 min) or Histoclear (15 min).

After 5 min in absolute methanol they were placed in 0.5% hydrogen peroxide in order to block any endogenous peroxidase present in the tissues (45 min). After rehydration and washing in distilled water, followed by Tris-buffered saline (TBS), the sections were transferred to 20% normal rabbit serum in TBS for 10 min. The primary antibody was applied in enclosed moist chambers at five drops per slide, and left at 4°C overnight. On the next day the slides were washed and the second

antibody applied: horseradish peroxidase-conjugated rabbit antimouse immunoglobulins (obtained from Dako Immunoglobulins, Denmark), diluted 1:30 in TBS, five drops per slide. Incubation was for 45 min at 37°C. After further washes in TBS, staining was developed in a 0.05% solution of 3,3'-diaminobenzidine hydrochloride (DAB) in Tris buffer, pH 7.6, with 0.01% hydrogen peroxide (20 min). Slides were washed and counterstained with Harris's haematoxylin, dehydrated, cleared and mounted.

An inappropriate monoclonal antibody (OX-6) was used as a negative control for each tumour, and a section of tumour known to express HMFG-1 on 90% of cells was used in each batch as a positive control.

Assessment of staining

Initial assessment was at low microscope power to examine the distribution of tumour cells and to note whether or not there were obvious gross variations in staining intensity. Intensity itself was recorded but as this variable is difficult to quantitate objectively, the intensity was not used in the final analysis. The number of cells which stained on each tumour section was expressed as a percentage of the total number of tumour cells counted. Each section had a minimum of four different areas counted. The site of staining was noted as being cytoplasmic (CY), on the cell membrane (CM) or a combination of both (CY +CM). In some tumours staining was found outside the cells in extracellular deposits (ECS), expressed as the number of deposits seen per high power (400×) field. A minimum of six fields was counted for each block, and the mean number used as the final figure. All slides were assessed by one observer (A.D.B.).

Statistical analysis

A chi-square analysis and Fisher's exact test were used for each comparison to relate frequencies of integers between two variables. In accordance with convention $P < 0.05$ was taken to be significant. Comparisons between two variables recorded as actual numbers (e.g. ER value with ECS deposits), were made with the Mann–Whitney U test, Pearson's correlation and Kendal's non-parametric correlation. Survival curves were calculated by the life table method. The log-rank test [13] and Cox regression analysis were used to compare the groups. Specificity and sensitivity for the accurate prediction of response to endocrine treatment were calculated for ER and PR concentrations and for the percentage of cells stained with HMFG-1. These results were plotted by receiver operating characteristic (ROC) curves for all three variables [14].

RESULTS

Response to endocrine therapy

Of the 168 patients, 20 (12%) had a complete response, 33 (20%) had a partial response, and 35 (21%) had static disease (Table 1). Response was highly significantly associated with survival from the start of treatment, and with time to progression on treatment. But there was no association between disease-free interval (DFI) and subsequent response to treatment. Becuase patients with static disease fared as well as those with a partial response, SD was considered to be a positive response to endocrine therapy, and SD is therefore included with PR and CR whenever the expression 'response' is used.

Histopathology

The major type of tumour was infiltrating duct carcinoma (IDC, 151, 89%). Eleven tumours (7%) were infiltrating lobular carcinoma (ILC), four were mucoid carcinoma (3%) and two were papillary carcinoma (1%). Grade was assessed in 141 of the 154 IDC tumours (93%): 22 (16%) were Grade I, 88 (62%) were Grade II and 31 (22%) were Grade III. Lymphatic or vascular invasion was assessed in 151 tumours: invasion was present in 48 (32%) and absent in 103 (68%). Elastosis was assessed in 148 tumours: it was present in 28 (19%) and absent in 120 (81%).

Histopathology and response to endocrine therapy

There was no association between the two major pathological types and response: 49% of patients with IDC responded, compared with 53% of those with ILC. Four of the five patients with mucoid carcinoma responded (80%), and both of the patients with papillary carcinoma responded.

Receptor proteins for oestradiol and progesterone

Reliable receptor analyses were available on 167 tumours. One hundred and eleven were oestrogen receptor positive (ER+, 66%), and 88 were progesterone receptor positive (PR+, 52%). There was a highly significant association between ER and response to endocrine treatment, and between PR and response: 68% of patients with ER positive tumours responded, compared with 18% of those with ER negative tumours ($P < 0.0001$), and 71% of those with PR postive tumours responded, compared with 29% of those with PR negative tumours ($P < 0.0001$) (Table 2). When ER and PR were considered together, with both present (ER+PR+) the response was 79%, and when neither was present (ER−PR−), only 17% responded (Table 2).

Table 2. Response according to oestrogen (ER) and progesterone (PR) receptor content of tumours

	n (%)	CR	PR	SD	CR+PR+SD (%)	PD (%)	
ER+	111 (66)	16	28	31	75 (68)	36 (32)	
ER−	56 (34)	3	4	3	10 (18)	46 (82)	
							$P < 0.0001$
PR+	87 (52)	14	22	26	62 (71)	25 (29)	
PR−	80 (48)	5	10	8	23 (29)	57 (71)	
							$P < 0.0001$
ER+PR+	79 (47)	14	21	26	61 (79)	18 (21)	
ER+PR−	32 (19)	2	6	6	14 (44)	18 (56)	
ER−PR+	8 (5)	1	1	0	2 (25)	6 (75)	
ER−PR−	48 (29)	2	4	2	8 (17)	40 (83)	
							$P < 0.0001$

Receptor status was unrelated to disease free interval from the time of mastectomy — the median DFI for patients with ER positive tumours was the same as that for those with ER negative tumours (22 months). The median DFI for those with PR postive tumours was 24 months, and for those with PR negative tumours 22 months.

Overall survival and survival from start of treatment for advanced disease was significantly greater in patients who had receptor positive tumours than in those with receptor negative ones (Fig. 1). The median survival from first presentation for patients with ER positive tumours was 58 months, compared with 45 months for those with ER negative tumour ($P < 0.04$), and the median survival from start of endocrine treatment was 30 months and 17 months respectively ($P < 0.0001$). There was a longer time to progression for patients with ER positive tumours compared with those who had ER negative tumours.

Immunostaining with HMFG-1

In order to determine the reproducibility of assessment, 47 sections were assessed on two occasions separated by a 3 month interval. In 40 of 47 (86%) the assessment of the percentage of cells stained was within 10% of the value estimated on the first occasion. In the tumours which had been assessed as ECS positive on the first reading, all were assessed as ECS positive on the second occasion, but there were minor differences in the number of deposits counted.

Of 168 tumours, 27 (16%) did not stain with HMFG-1, and 141 expressed stain on a range of cell percentages from 1 to 90% (mean value 30%, median, 31%, Table 3). Cytoplasm alone (CY), was

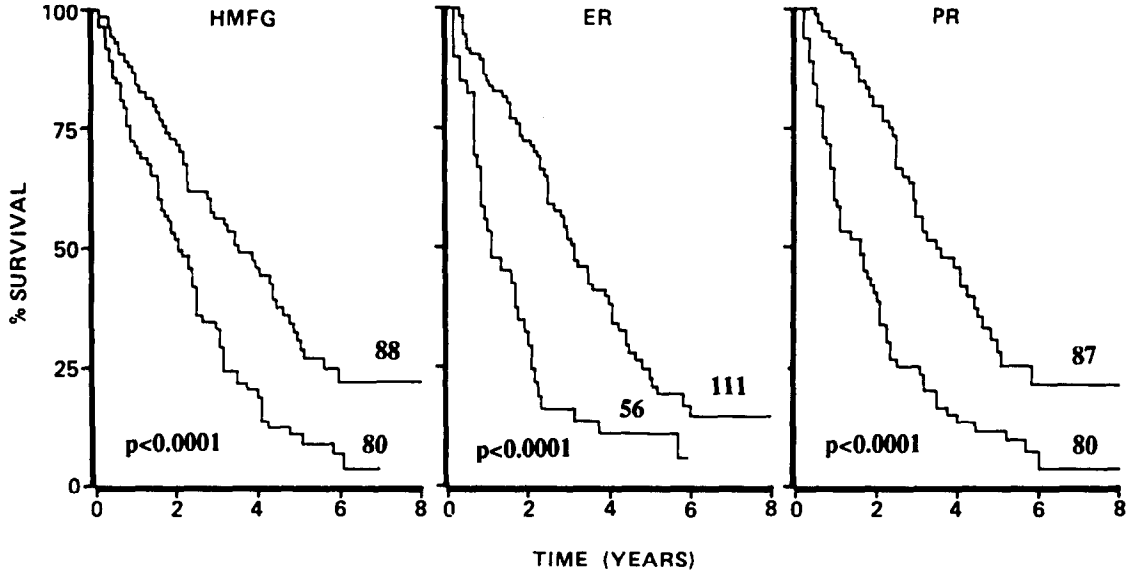

Fig. 1. Survival from the start of endocrine treatment related to the expression of HMFG-1, ER and PR. For HMFG-1, ≥20% cells stained was regarded as positive, and for ER and PR, ≥5 fM/mg cytosol protein was regarded as positive. Upper curves are tumours which were positive for each phenotype: lower curves are tumours which were negative for each phenotype.

Table 3. Percentage of cells stained with HMFG-1 and response

	n (%)	CR	PR	SD	CR+PR+SD	PD
Nil	27 (16%)	0	3 (11%)	4 (15%)	7 (26%)	20 (74%)
1–9%	34 (20%)	2 (6%)	2 (6%)	5 (15%)	9 (27%)	25 (73%)
10–19%	19 (11%)	1 (3%)	3 (8%)	3 (8%)	7 (37%)	12 (63%)
20–29%	19 (11%)	2 (5%)	2 (5%)	6 (16%)	10 (53%)	9 (47%)
30–49%	20 (12%)	2 (10%)	7 (35%)	6 (30%)	15 (75%)	5 (25%)
50–100%	49 (29%)	13 (27%)	15 (31%)	10 (21%)	38 (79%)	11 (21%)
		Chi-square = 43.903, $P < 0.0001$				
CM present	60 (36%)	11 (18%)	19 (32%)	11 (18%)	41 (68%)	19 (32%)
		Chi-square = 16.830, $P < 0.0008$				
ECS present	29 (18%)	5 (17%)	10 (38%)	7 (24%)	22 (76%)	7 (24%)
		Chi-square = 7.387, $P < 0.007$				

stained in 81 (48%), cell membrane alone (CM), in 16 (9.5%), and both CY and CM in 44 (24%). Extracellular deposits (ECS) were found in 29 tumours (18%).

The relationship between response to endocrine therapy and the percentage of cells stained was highly significant ($P < 0.0001$, Table 3). When more than 30% of tumour cells stained, 53 of 69 (77%) responded to treatment, and 37 of these 53 (70%) had complete or partial responses. When less than 10% of tumour cells stained, only 16 of 61 (26%) responded. Patients with tumours that did not stain at all fared particularly badly: 20 of 27 (74%) had progression of disease. The proportion of cells stained was greater in responders than in non-responders. The median value for patients with CR was 65%, with PR 37%, with SD 20%, and with progression 8%. Survival from the start of treatment was significantly longer for patients whose tumours stained ≥20% cells than for those whose tumours showed a lower degree of stain or none at all ($P < 0.0001$, Fig. 1).

The presence of ECS also related significantly to response (chi-square=7.387, $P < 0.007$), as did the presence of cell membrane staining (chi-square=16.830, $P < 0.0008$) (Table 3).

There was a strong association between the expression of HMFG-1 and steroid hormone receptor concentrations. When the percentage of cells stained was related to the concentrations of ER and PR (in fM/mg cytosol protein) by means of Kendal's non-parametric correlation, there were highly significant associations (for ER, $\tau = 0.19$, $z = 3.54$, $P < 0.001$; for PR, $\tau = 0.18$, $z = 3.48$, $P < 0.001$).

The percentage of cells stained was related to tumour grade (chi-square = 21.05, $P < 0.007$), and to the presence of elastosis (chi-square = 10.98, $P < 0.03$). The median percentage of cells stained for Grade I tumours was 68%; for Grade II tumours this was 17%, and for Grade III tumours 9%. There was no association between the percentage of cells stained and individual components of grade, nor with the presence or absence of lymphatic invasion.

Multivariate analysis

Multivariate analysis was performed in order to determine the relative importance of the factors considered in the preceding sections for the prediction of survival from the start of endocrine therapy. These were related to other factors of possible prognostic significance such as tumour size, Karnofsky performance status, dominant site of disease, and biochemical variable such as liver enzymes and serum albumen. Firstly log-rank analyses were performed. Response and receptor status were the most significant factors for survival from the start of treatment. When all these factors were entered in a Cox multivariate analysis, response was the most important prognostic indicator ($P < 0.00001$). However, the response is not known at the time of the start of treatment, and therefore the data were analysed again but with response excluded. PR was the most important prognostic indicator. The analysis was repeated, excluding both response and receptor data. The best predictor of survival from endocrine treatment was the percentage of cells stained with HMFG-1: high expression (≥20% cells stained) was associated with a longer survival than that found for patients with less stained and non-stained tumours ($P = 0.00053$). The four next features which were found to relate to survival were serum alkaline phosphatase ($P = 0.0075$), the Karnofsky performance status ($P = 0.00065$), the presence of cell membrane stain with HMFG-1 ($P = 0.0090$), and the presence of soft tissue disease as the dominant site of relapse ($P = 0.020$).

ROC curves

To investigate whether HMFG immunostaining could substitute for ER and PR for the prediction of response to endocrine treatment, receiver operating characteristic (ROC) curves were plotted for all three variables [14] (Fig. 2). Sensitivity and speci-

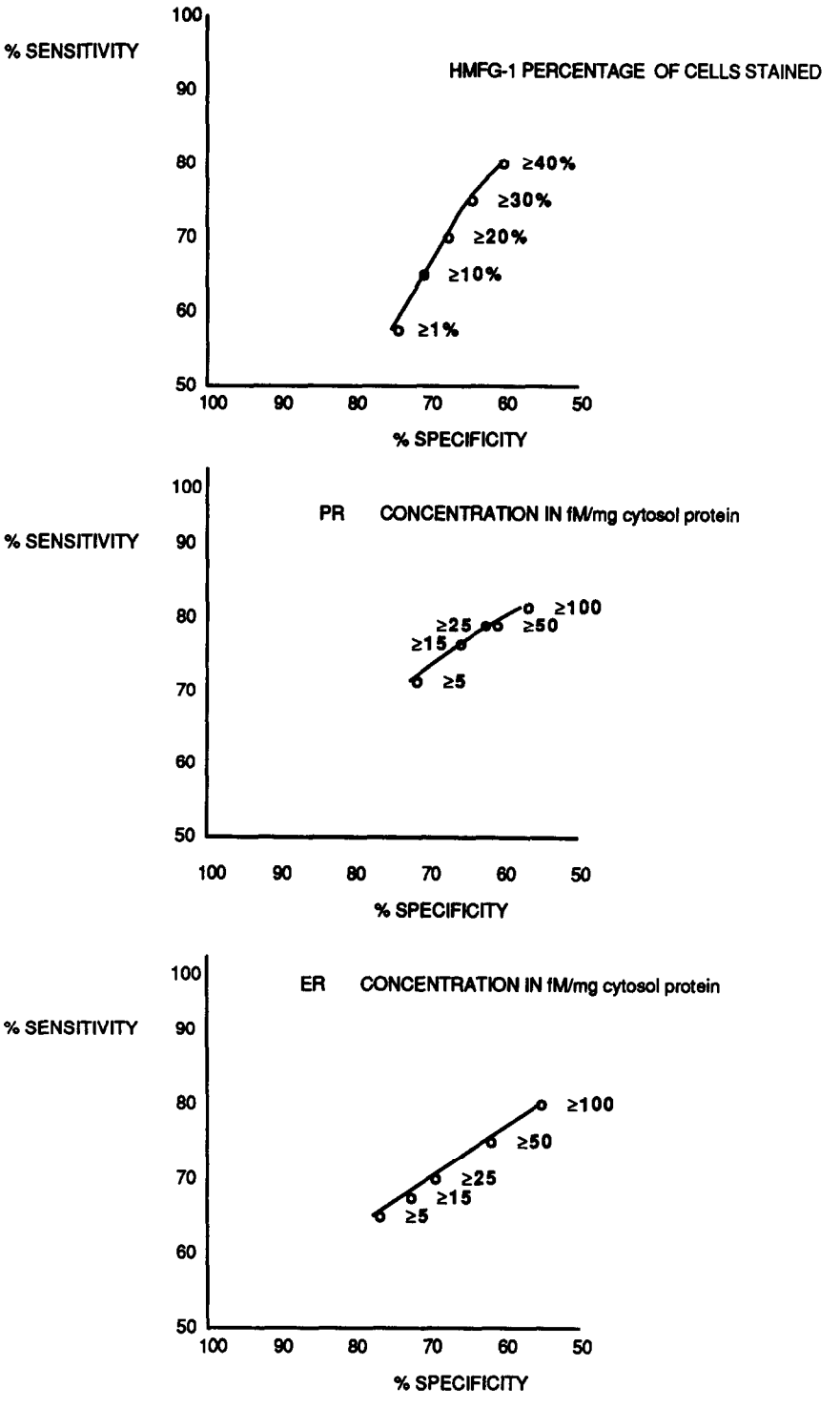

Fig. 2. Sensitivity and specificity for the prediction of response—ROC curves for HMFG-1, progesterone (PR) and oestrogen (ER) receptors.

ficity for prediction of response were calculated over a wide range of values for ER and PR, and over a wide range of percentage of cells stained for HMFG-1. A cut-off threshold of ⩾5 fM/mg cytosol protein gave optimum sensitivity (72%) and specificity (70%) for PR: optimum findings for ER were at a threshold of 30 fM (sensitivity 77%, specificity 68%). HMFG-1 gave very similar findings if a threshold of ⩾20% cells stained was used: sensitivity for prediction of response was 71%, specificity 68%. From these results HMFG-1 was as accurate for the prediction of response as ER and PR.

DISCUSSION

Several monoclonal antibodies raised to cell-surface antigens have been studied in cancer of the breast [15–19]. In this study the proportion of cells stained with HMFG-1 was associated with response to endocrine treatment, histological differentiation and oestrogen and progesterone receptors.

In the lactating breast antigen expression is found within the cytoplasm, on the cell membrane, and within the lumina of ductules [5, 6]. The antigens are expressed to the greatest extent during lactation, and may be regarded as differentiation antigens. This is supported by *in vitro* studies [20, 21]. In cultured normal milk epithelial cells, HMFG-1 is expressed when the cells become differentiated [20]. Other groups using HMFG-1 have also shown an association of staining with grade [7, 6, 16].

With HMFG-1, there was a highly significant correlation between the degree of antigen expression and oestrogen receptor concentration ($P < 0.001$), and with progesterone receptor concentration ($P > 0.001$). This is consistent with other papers which report an association between the degree of differentiation as determined by histological grade and receptor status [22–29]. Tumours with a low grade and high receptor content are likely to respond to endocrine therapy. It is not surprising that antigen expression correlates with response to endocrine treatment. When $\geq$20% of cells stained the correlation with response was as accurate as that found by measurement of oestrogen and progesterone receptors.

HMFG-1 did not give an indication of the disease-free interval of this group of patients, but all were selected on the basis that relapse had already taken place, or the primary disease was advanced at the time of treatment. This is similar to results obtained by us and others for the lack of association between receptor status and disease-free interval [4, 25, 30–34]. If disease-free interval is related to the rate of growth of the tumour, then neither HMFG antigens nor receptors give an indication of growth rate. Their relationship to overall survival and survival after relapse is related to indication of response to endocrine therapy.

The presence of progesterone receptor within tumours was the single most important prognostic factor for survival from the start of endocrine therapy. This is presumed to be because PR indicates response to treatment, rather than a feature of PR irrespective of response. We have found that the PR negative responders have the same survival characteristics as PR positive repsonders [32]. In contrast PR positive non-responders have the same survival characteristics as PR negative non-responders [32]. These data suggest that response is a more important prognostic indicator than PR. Like PR, HMFG-1 positive (i.e. $\geq$20% cells stained) non-responders have a poor prognosis: thus HMFG-1 is also probably an indicator of prognosis by virtue its indication of response to endocrine therapy.

HMFG-1, ER and PR may be useful for treatment stratification. With all three a high degree of sensitivity (positive value associated with positive response) is gained at the expense of loss of specificity (negative value being associated with response). It is customary to divide ER and PR into positive and negative at an arbitrary value of about 5–15 fmols/mg of cytosol protein. At these thresholds the sensitivity and specificity for each receptor is 70%: an equivalent cut off for HMFG-1 is at 20% of cells stained. The similarity of the ROC curves suggest that receptors and HMFG-1 expression measure similar tumour features. But experiments where adjacent sections of tumour are stained for ER and HMFG-1 with monoclonal antibodies indicate that different cell populations stain in some cases. Some cells express ER but not HMFG-1, and vice versa.

The antigens recognised by HMFG-1 are probably oligosaccharide sequences [2]. We and others have found that the molecular weight of epitopes which can be demonstrated from homogenized cell preparations run on Western blots is highly variable [2, 3]. The antigen found in human milk is approx. 400 K molecular weight. Tumours which contain these higher molecular weight components are more likely than others to be receptor positive and to express cell membrane and extra-cellular staining [3]. This suggests that some tumours can produce similar components to those found in milk, and in the same distribution as that found in the lactating breast. These are the tumors which are most likely to respond to endocrine treatment and have the best prognosis.

Heterogeneity has been reported with virtually all monoclonal antibodies raised against membrane-preparations of tumours or milk fat globule membranes [15]. In the resting normal breast staining is also heterogeneous [5, 12, 19]. Estimation of percentage of cells is highly reproduceable and does not appear to be markedly affected by heterogeneity within each tumour: this is in agreement with other studies [6, 7, 16, 17]. When two consecutive sections were stained on separate occasions the staining pattern was reproduceable, but intensity of staining often differed. Intensity was not used in these analyses.

These results are encouraging with regard to a possible role for HMFG-1 staining in analysis of tumours. The technique is relatively simple to perform, does not require fresh tissue and can be used on paraffin-embedded samples of tumour. Sections of primary tumour from several years previously can be used in patients who present with inaccesible recurrent disease. Interpretation of staining is a problem which needs to be addressed further, but until more objective means of interpretation are developed, the percentage of cells stained and the presence or absence of ECS and CM stain give useful information. Immunoperodixase staining with HMFG-1 is commended as an alternative to receptor assays whenever fresh tissue is not available.

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