DISEASE MARKERS, VOL. 11, 91–101 (1993)

IMMUNOHISTOCHEMICAL DISTRIBUTION OF A BREAST CANCER-ASSOCIATED GLYCOPROTEIN

P.D. RYE* AND R.A. WALKER
University of Leicester, Breast Cancer Research Unit, Clinical Sciences, Glenfield General Hospital, Groby Road, Leicester LE3 9QP, UK.

SUMMARY

The tissue distribution and specificity of a glycoprotein of M_r 230 000kDa which has previously been identified from breast carcinomas in culture and shown to be tumour-associated, has been assessed using a polyclonal antiserum. A wide range of tissues has been examined immunohistochemically. The tissue distribution of the glycoprotein show differences between normal, benign and malignant breast and other epithelial tissues, and are clearly specific for epithelial cells. This glycoprotein as detected by the polyclonal antiserum P5252-2, was either absent or showed a minimal presence in normal breast tissues. Evidence of the expression of the glycoprotein in hyperplastic breast was observed but was considerably less than that seen for carcinomas, for which 70% had greater than 50% of cells exhibiting reactivity with P5252-2. There was no relationship with grade or node status. Similar striking differences in glycoprotein expression between non-neoplastic and neoplastic tissue were observed for stomach, large intestine, thyroid and to lesser extent ovary. The differences in the expression of this glycoprotein between normal and malignant tissues is of obvious clinical and pathological potential.

KEY WORDS Breast Cancer Glycoprotein Immunohistochemistry Tumour Marker

INTRODUCTION

Glycosylation changes in malignancy have become a well recognised phenomena (Warren and Buck, 1980; Smets and Van Beek, 1984; Hakomori, 1985). The role of glycoconjugates in normal cell interactions suggests that specific alterations in glycoconjugate expression associated with malignancy could be significant in determining tumour cell behaviour. This has resulted in the search for tumour-associated glycoconjugates of biological and clinical interest.

The introduction of monoclonal antibody technology has facilitated this search and in the human breast a variety of different immunogens have been used including milk fat globule membranes (Taylor-Papadimitriou *et al.*, 1981; Foster *et al.*, 1982; Hilkens *et al.*, 1984), breast cancer cell lines (Papsidero *et al.*, 1983; Thompson *et al.*, 1983; White *et al.*, 1985; Mesa-Tejada *et al.*, 1988), and membrane enriched extracts of human metastatic mammary carcinomas (Colcher *et al.*, 1981; Kufe *et al.*, 1984; Ellis *et al.*, 1984). However, a significant disadvantage of the immunological approaches used thus far, has been their selectivity for the more immunogenic components of the polymorphic epithelial mucins or PEM's (Griffiths *et al.*, 1987; Taylor-Papadimitrou and Gendler,

*Correspondence to: Dr P.D. Rye, University of Leicester, Breast Cancer Research Unit, Clinical Sciences, Glenfield General Hospital, Groby Road, Leicester LE3 9QP, UK.

© 1993 Asfra BV
Received 2 November 1992
Revised 17 January 1993

1988). In spite of this problem few workers have attempted to identify potentially useful markers by non-immunogenic methods.

In a study analysing the glycoproteins released by non-malignant and malignant human breast, we identified a considerably higher incidence of glycoproteins in the molecular weight range M_r210 000kDa to M_r280 000kDa from carcinomas than benign samples (Rye and Walker, 1989). In particular a fucosylated glycoprotein of M_r230 000kDa was identified from 65% of carcinomas but no benign tissues. This clearly has potential as a tumour-associated marker, which could provide prognostic information. To assess the significance and distribution of this glycoprotein, a polyclonal antiserum has been generated and screened against a wide variety of normal and malignant tissues, including breast.

MATERIALS AND METHODS

Production of Antisera

Media derived from twelve primary breast carcinomas which had undergone organ culture for 48 hours were pooled. Previous analysis using [^{3}H] fucose tracer had determined that the medium contained the fucosylated glycoprotein of M_r230 000kDa. The pooled sample totalling 800µg of total protein as determined by the method of Bradford (1976) was applied to a 7.5% SDS polyacrylamide gel using the discontinuous buffer system of Laemmli, (1970). Two check lanes for the sample and molecular weight standards were included in the 4% stacking gel. After electrophoresis the two check lanes were processed for fluorography and the remaining gel was stored at –20°C until the fluorographic record was obtained. From the molecular weight estimations the strip of gel containing the glycoprotein M_r230 000kDa was excised and minced finely in 0.5ml saline in preparation for immunization. Glycoprotein purity was re-checked by SDS-polyacrylamide gel electrophoresis prior to immunization.

Three male New Zealand White rabbits were used. After adding 1.5ml of Freunds complete adjuvant (FCA) to the minced gel fragments, the mixture was emulsified and administered by intramuscular injection, 0.25ml into each hind leg of all three animals. At 14 and 32 days after primary immunization further injections were given but using Freunds incomplete adjuvant. Test bleeds were taken prior to injection. Total serum Ig was monitored using the SIRD method of Mancini (1976).

Immunoblotting

Media from organ cultures of carcinomas cultured as above (Rye and Walker, 1989) were separated electrophoretically on SDS polyacrylamide gels as outlined above. Transfer onto nitrocellulose membranes was performed as described by Towbin *et al.*, (1979) using the Biorad Transblot apparatus for 16 hours at 300mA fixed current, in 25mM Tris, 192mM glycine buffer, pH8.3, with the exception that methanol was omitted. The nitrocellulose was blocked for 1 hour at 37°C with 0.3% Tween-20 (Batteiger *et al.*, 1982) containing 20% swine serum. The polyclonal antiserum P5252-2 was incubated for 2 hours at room temperature. After three 10 minute washes in PBS containing 0.05% Tween-20 the nitrocellulose was incubated with peroxidase labelled swine anti rabbit immunoglobulin antiserum. The peroxidase was subsequently localised using diaminobenzidine-hydrogen peroxide.

Immunohistochemistry

Twenty one normal and benign breast samples and 68 carcinomas were examined, as well as 150 other tissue types of normal, benign and malignant origin. All samples had been fixed in 4% formaldehyde in saline for 18 to 36 hours and processed through to paraffin wax. After dewaxing and rehydration endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes. Sections were washed and non-specific binding blocked with 20% normal swine serum for 10 minutes. The primary antiserum P5252-2 was applied at 1:200 and 1:500 dilution (11.25μg/ml and 4.5 μg/ml respectively, total rabbit Ig), for 2 hours at room temperature. After washing in PBS peroxidase swine anti rabbit immunoglobulin antiserum was applied. Peroxidase was localised using diamino-benzidine-hydrogen peroxide, and nuclei counterstained with Meyers Haematoxylin. Omission of primary reagent as a negative control, and breast carcinoma tissue of known positive reactivity to P5252 as a positive control were included throughout the study.

Histology

Breast carcinomas were classified according to WHO criteria and graded using the modified Bloom and Richardson system (Elston, 1987). Staining was categorized as negative; <25% positive cells; 25–50% cells reacting; 50–75% cells stained; and >75% positive cells. Staining was assessed individually by both authors, with good agreement (>95%). Where differences occurred staining was reassessed and categorized after discussion.

RESULTS

A number of different epithelial tissues studied showed reactivity with the antiserum P5252-2. The antiserum was specific for epithelial cells and showed no reactivity with plasma cells, stromal tissue or other non-epithelial cells. Immunoblotting with P5252-2 identified a broad band of apparent M_r230 000kDa on SDS discontinuous polyacryl-amide gel electrophoresis (Figure 1), which corresponds to the purified fucosylated N-linked glycoprotein used as the immunogen, that has been previously identified (Rye and Walker, 1989).

Immunohistochemistry of Normal and Benign Breast

There were 15 samples which contained normal lobules and ducts along with varying degrees of blunt duct adenosis, mild regular ductal hyperplasia and apocrine metaplasia. In seven there was no evidence of staining of normal elements. The other eight showed luminal staining (<25% cells) of acinar and ductal epithelium although in two cases staining of ductal epithelium only was seen. There was luminal staining of all cells showing apocrine metaplasia. Luminal cell staining was seen in hyperplastic glands and ducts, ranging in frequency from <10% of structures showing that change to around 50%, with a median of 20%.

One sample of breast from a 20 week pregnant woman was examined and this showed luminal staining of secretory-type glands with no reactivity of acini not showing secretory-type change. Tissue from lactating breast showed luminal and cytoplasmic staining of all glands, and secretions.

There were four fibroadenomas, one of which showed pregnancy change. The intracanalicular-type ducts showed luminal staining in >75% of the epithelium, whereas

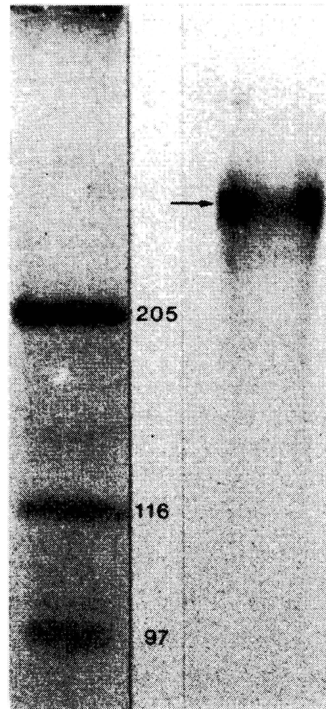


Figure 1. Immunoblotting of media from a cultured breast carcinoma showing reactivity of P5252-2 with the glycoprotein M_r230 000kDa (arrowed). Adjacent lane shows molecular weight markers.

the pericanalicular-type ducts had luminal staining of <25% of the epithelium. The pregnancy changes showed similar reactivity to that already described.

Breast Carcinomas

The overall findings in relation to type, grade and node status are shown in Table 1. There were two carcinomas with no evidence of staining (3%); 7 cases (7%) with <25% cells reacting; 12 cases (18%) showing 25–50% cells staining; 21 cases (31%) with 50–75% positive cells, and 26 cases (38%) showing >75% cells staining. Overall the frequency of staining was greater than that seen in hyperplastic breast, with 47/68 cases (69%) having >50% positive cells. The differences observed between adjacent areas of normal and malignant glandular epithelium are shown in Figure 2. A difference in localisation of staining was also observed, in that luminal reactivity was only seen in areas of tubular differentiation, and the predominant reactivity was focal or diffuse cytoplasmic, with or without membrane accentuation, along with staining of intra-cytoplasmic lumina (ICL), as shown in Figure 3.

The majority of cases were infiltrating ductal carcinomas. Four of the five infiltrating lobular carcinomas had more than 50% of cells reacting. Using 50% staining as the dividing point, and grouping well and moderately differentiated carcinomas together due to the small numbers, there was no significant association between staining and differentiation ($0.1>P>0.05$; $\chi^2 3.82$; 1d.f.) or nodal status ($0.5>P>0.1$; $\chi^2 1.41$, 1d.f.).

Table 1. Extent of reactivity of antibody P5252-2 in relation to breast carcinoma type, differentiation and node status

Histology	Neg	<25%	25-50%	50-75%	>75%
Type					
Infiltrating ductal	2	6	10	18	23
Infiltrating lobular			1	1	3
Mucinous			1	1	
Secretory				1	
Medullary		1			
Grade					
Well differentiated		1		1	1
Moderately differentiated		2	5	8	20
Poorly differentiated	2	4	7	12	5
Node Status					
Metastasis	1	2	7	16	15
Free from disease	1	3	4	3	8
Status not known		2	1	2	3
Total No. Cases	2	7	12	21	26

Other Tissues

The overall results are shown in Table 2. In the *gastrointestinal system* staining was seen in the parietal cells of normal stomach. One sample of normal large intestine showed staining of the glycocalyx of surface epithelium but not of glands but the remaining seven cases were negative, as was a case of tubular adenoma. The three oesophageal carcinomas were all adenocarcinomas and showed prominent reactivity, as did all gastric carcinomas and five of the seven large intestinal carcinomas. In one of the sections of stomach there was a striking transition in staining between normal glands and those showing carcinoma-*in-situ* (Figure 4).

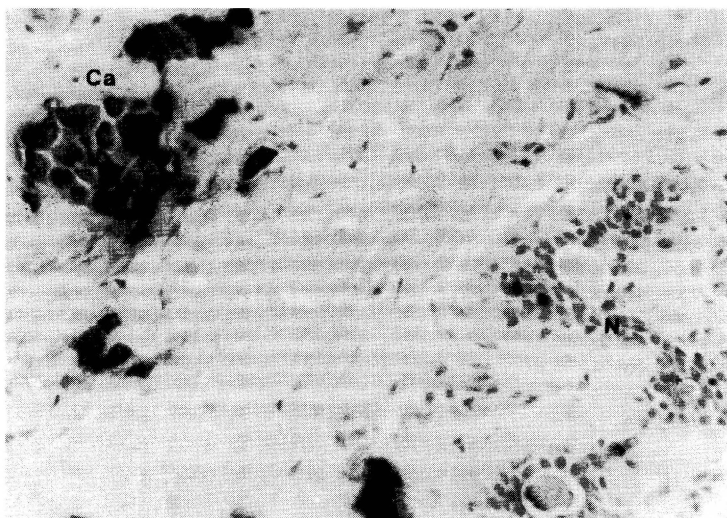


Figure 2. Breast carcinoma showing differences in staining with P5252-2 between normal acini structures (N) and tumour cell islands (Ca).

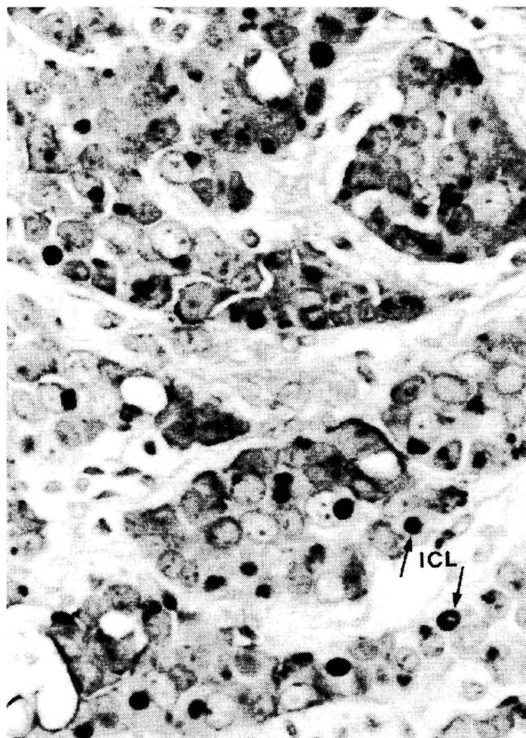


Figure 3. High power view of breast carcinoma showing focal cytoplasmic reactivity with P5252-2 Note staining of intracytoplasmic lumina, (ICL).

Table 2. Reactivity of P5252 with tissues of normal and malignant origin

Tissue Type	Normal					Malignant				
	Neg	<25%	25-50%	50-75%	>75%	Neg	<25%	25-50%	50-75%	>75%
Gastrointestinal Tract										
Salivary Gland	1	2				nt				
Oesophagus	1								3	
Stomach		6[a]							6	
Appendix	3	1				nt				
Large Intestine	7	1					2		5	
Gall Bladder	3					nt				
Liver	4	1				1				
Genito Urinary Tract										
Kidney - glomeruli[b]	2									
tubules				2		nt				
Testis	5					2				
Epididymis			3			nt				
Vas deferens		1				nt				
Prostate	2						8			1
Ovary	4					3			3	
Fallopian Tube	5	1				nt				
Endometrium			8						2	
Myometrium	1					nt				
Endocervix			4				4			1
Ectocervix	4					1				
Endocrine										
Parathyroid	2					nt				
Thyroid	3	2							1	
Adrenal	1					1				
Lung		3[c]	1[c]			1	1	2	2	
Skin										
Epidermis	4	2				1	1		1	
Skin Appendage			3			nt				
Lymphoreticular										
Lymph node	4					2				
Connective Tissue										
Nerve	2					3				
Fat	2					3				

nt Malignant tissues not tested
a Staining only observed in parietal cells of stomach
b No malignancy of kidney glomerulus
c Normal lung tissues were obtained from lobectomies or pneumonectomies for cancer

In the *genito-urinary system* staining of the luminal surface of tubular epithelium was observed. Staining was also seen of the luminal surface of epididymal ducts, endometrial glands irrespective of the phase of the cycle, and endocervical glands. Endometrial carcinomas showed reactivity but the extent of staining of endocervical adenocarcinomas was less than the normal glands. Only one of five samples of fallopian tube showed staining and this was involved by endometriosis. Three of six ovarian carcinomas showed prominent reactivity. There was no staining of stromal components. Testis and testicular tumours were negative. Normal prostate was negative whereas all carcinomas showed staining although only one had prominent reactivity.

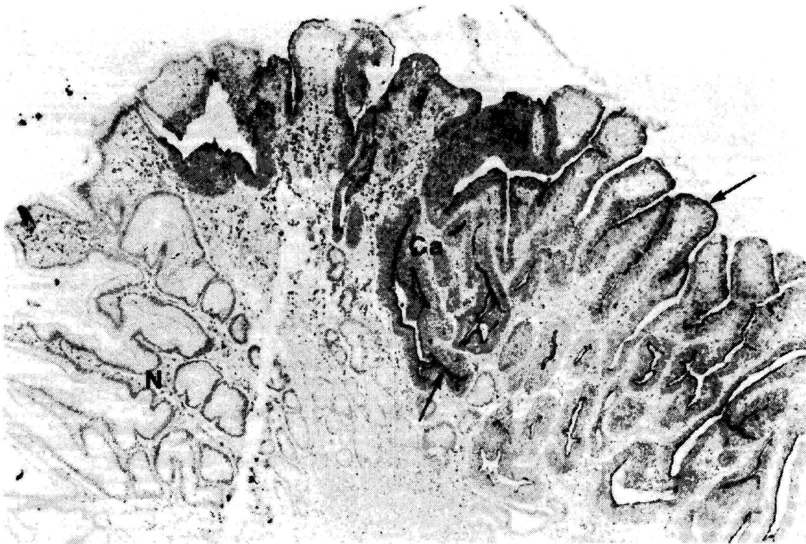


Figure 4. Stomach showing differences in staining with P5252-2 between normal surfaces and glandular epithelium (N), and carcinoma *in situ* (Ca). Note strong staining of glycocalyx and luminal borders of abnormal glands (arrowed)

The *endocrine system* generally showed little reactivity but in one case there was prominent staining of a follicular carcinoma while the adjacent normal tissue was virtually negative (Figure 5).

Obtaining entirely normal *lung* was difficult. Staining of reactive alveolar cells was seen, all specimens being from lobectomies/pneumonectomies for cancer. An oat cell carcinoma was negative; the adeno-carcinomas and squamous cell carcinomas showed differing degrees of reactivity.

In the *skin* there was staining of the luminal surface of sweat glands, and areas of hyperplastic but not normal squamous epithelium. Malignant melanoma was negative, and the two squamous cell carcinomas showed varying degrees of reactivity.

There was no staining of neoplasms of lymph nodes, fat or nerves.

DISCUSSION

We have shown in a wide range of tissues the distribution of the high molecular weight glycoprotein (M_r230 000kDa), previously identified by us as tumour-associated (Rye and Walker, 1989). Our findings indicate that the expression of this glycoprotein, as detected by the polyclonal antiserum P5252-2, is clearly specific to epithelial cells. Moreover, differences in its distribution between normal, benign and malignant epithelial tissues supports our previous studies suggesting that the glycoprotein is tumour associated. Indeed there were some striking differences in the reactivity of the P5252-2 antiserum between normal epithelium and carcinomas of breast, stomach, large intestine, thyroid and to a lesser extent ovary. Whilst this indicates that the glycoprotein identified by the antibody is not breast-specific it emphasises its association with malignancy in these tissues, which could be of diagnostic value. Such an association however, was not found for certain parts of the genital tract.

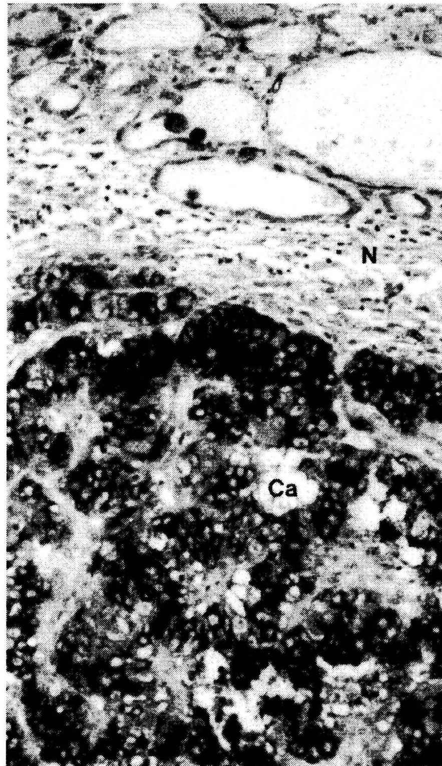


Figure 5. Follicular carcinoma of the thyroid with areas of normal tissue (N) having little evidence of staining with P5252-2 and adjacent carcinoma (Ca) showing prominent staining

In normal breast, reactivity of P5252-2 was either completely absent or present at low levels, although the frequency increased with hyperplastic change, and was always seen with apocrine metaplasia and lactational change. The extent of the staining in carcinomas was much greater than in benign breast tissue and showed significant differences in staining localization. The alteration in localization from luminal to cytoplasmic has also been described for many other antibodies (Ellis *et al.,* 1984; Arklie *et al.*, 1981) and may be due to a failure in transport to the cell surface (Corcoran and Walker, 1990). There was no association between extent of staining of the carcinomas and differentiation, which is similar to the findings for several milk fat globule membrane antibodies with the exception of NCRC-11 (Walker, 1990). Some of the staining patterns observed in various tissues with the polyclonal antiserum were similar to those described for a variety of monoclonal antibodies generated against milk fat globule membrane and breast cancer cell lines (Hilkens *et al.*, 1984; Arklie *et al.*, 1981). However, the extent and distribution of staining in breast carcinomas differed from the patterns observed with many of these antibodies (Walker, 1990). It is evident that a number of the antibodies previously generated, whether against milk fat globule membrane, isolated breast glycoproteins or breast cancer cell lines, detect different but related components of PEM (Taylor-Papadimitriou and Gendler, 1988). Whilst this may be true for some of those antibodies mentioned, earlier studies using the N-linked glycosylation inhibitor tunicamycin, have

identified the glycoprotein of M_r230 000kDa to be N-linked (Rye, unpublished data) and therefore differ from those previously described as PEM.

The antiserum generated has confirmed that for breast and certain other tissues the glycoprotein of M_r230 000kDa previously identified is tumour associated and of potential clinicial value. Further study using monospecific reagents to this glycoprotein and to its deglycosylated peptide core may provide additional evidence supporting its role in malignancy.

ACKKNOWLEDGEMENTS

We are grateful to the Cancer Research Campaign and Trent Regional Health Authority for financial support, and Mrs S. Dearing for technical assistance.

REFERENCES

Arklie, J., Taylor-Papadimitriou J., Bodmer, W., Egan, M. and Millis, R. (1981). Differentiation antigens expressed by epithelial cells in lactating breast are also detectable in breast cancer. *Int. J. Cancer*, **28**, 23–30.

Batteiger, B., Newhall, W.J. and Jones, R.B. (1982). The use of tween-20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *J. Immun. Methods*, **55**, 297–307.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248–254.

Colcher, D., Horan-Hand, P., Nuti, M. and Schlom, J. (1981). A spectrum of monoclonal antibodies reactive with human mammary tumour cells. *Proc. Natl. Acad. Sci. USA*, **78**, 3199–3202.

Corcoran D. and Walker, R.A. (1990). Ultrastructural localization of milk fat globule membrane antigens in human breast carcinomas. *J. Pathol.*, **161**, 161–166.

Ellis, I.O., Robbins, R.A., Elston, C.W., Blamey, R.W., Ferry, B. and Baldwin, R.W. (1984). A monoclonal antibody NCRC-11 raised to human breast carcinoma: Production and immunohistological characterization. *Histopathol.*, **8**, 501–516.

Ellis, I.O., Bell, J., Todd, J.M., Williams, M., Dowle, C., Robbins, R.A., *et al.* (1987). Evaluation of immunoreactivity with monoclonal antibody NCRC 11 in breast carcinoma. *Br. J. Cancer.*, **56**, 295–299.

Elston, C.W. (1987). Grading of invasive carcinoma of the breast. In: Page, D.L. and Anderson T.J. (Eds). *Diagnostic Histopathology of the Breast.* Edinburgh: Churchill Livingston, 300–311.

Foster, C.S., Edwards, P.A.W., Dinsdale, E.A., Neville, A.M. (1982). Monoclonal antibodies to the human mammary gland: Distribution of determinants in non-neoplastic mammary and extramammary tissues. *Virchows. Arch. (Pathol. Anat.)*, **394**, 279–293.

Griffiths A.B., Burchell, J., Gendler, S., Lewis, A., Blight, K., Tilly, R. *et al.* (1987). Immunological analysis of mucin molecules expressed by normal and malignant mammary epithelial cells. *Int. J. Cancer,* **140,** 319–327.

Hakomori, S-I. (1985). Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. *Cancer Res.*, **45**, 2405–2414.

Hilkens, J., Buijs, F., Hilgers, J., Hageman, Ph., Calafat, J., Sonnenberg, A. *et al.* (1984). Monoclonal antibodies against human milk fat globule membranes detecting differentiation antigens of the mammary gland and its tumours. *Int. J. Cancer*, **34,** 197–206.

Hudson, L and Hay, F. (1976). *Practical Immunology.* Oxford: Blackwell Scientific.

Kufe, D., Inghirami, G., Abe, M., Hayes, D., Justi-Wheeler, H. and Schlom, J. (1984). Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumours. *Hybridoma,* **3**, 223–232.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Mesa-Tejada, R., Palakodety, R.B., Leon, J.A., Khatcherian, A.O. and Greaton, C.J. (1988). Immunocytochemical distribution of a breast carcinoma associated glycoprotein identified by monoclonal antibodies. *Amer. J. Pathol.*, **130,** 305–314.

Papsidero, L.D., Nemoto, T. and Ming Chu, T. (1983). Monoclonal antibodies (F36/22) and M7/105) to human breast carcinoma. *Cancer Res.*, **43**, 1741–1747.

Rye, P.D. and Walker, R.A. (1989). Analysis of glycoproteins released from benign and malignant human breast: changes in size and fucosylation with malignancy. *Eur. J. Cancer Clin. Oncol.*, **25,** 65–72.

Smets, L.A. and van Beek, W.P. (1984). Carbohydrates of the tumour cell surface. *Biochim. Biophys. Acta,* **738**, 237–249.

Taylor-Papadimitriou, J., Arklie, J., Burchell, J., Ceriani, R.L. and Bodmer, W.F. (1981). Monoclonal antibodies to epithelial-specific component of the human milk-fat globule membrane: production and reaction with cells in culture. *Int. J. Cancer,* **28**, 17–21.

Taylor-Papadimitriou, J. and Gendler, S.J. (1988). Molecular aspects of mucins. *Cancer Rev*, **11/12**, 17–24.

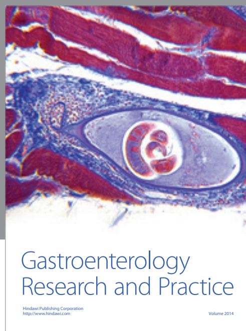
Thompson, C.H., Jones, S.L., Whitehead, R.H. and MacKenzie, I.F.C. (1983). A human breast tissue-associated antigen detected by a monoclonal antibody. *J. Natl. Cancer Inst.*, **70**, 409–419.

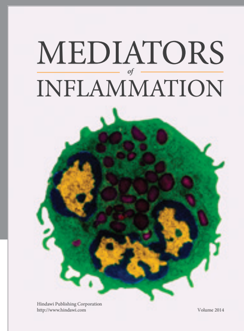
Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA,* **76**, 4350–4354.

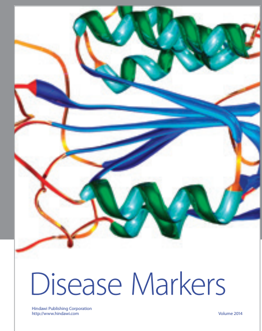
Walker, R.A. (1990). Assessment of milk fat globule membrane antibodies and lectins as markers of short-term prognosis in breast cancer. *Br. J. Cancer,* **63,** 462–466.

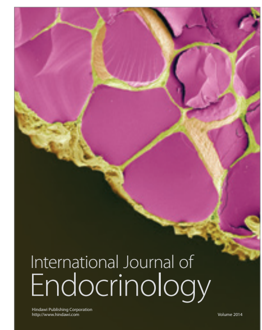
Warren, L. and Buck, C.A. (1980). The membrane glycoproteins of the malignant cell. *Clin. Biochem.*, **13**, 191–197.

White, C.A., Dulbecco, R., Allen, R., Bowman, M. and Armstrong, B. (1985). Two monoclonal antibodies selective for human mammary carcinoma. *Cancer Res.*, **45**, 1337–1343.









Hindawi

Submit your manuscripts at
http://www.hindawi.com