Pergamon

European Journal of Cancer Vol. 32A, No. 9, pp. 1585–1590, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

PII: S0959-8049(96)00116-5

Original Paper

pS2 Protein Expression in Gastric Carcinoma. An Immunohistochemical and Immunoradiometric Study

J.-C. Machado,[1] F. Carneiro,[1] P. Ribeiro,[2] N. Blin[3] and M. Sobrinho-Simões[1]

[1]Department of Pathology, IPATIMUP, Medical Faculty, University of Porto, H.S. João, 4200 Porto, Portugal; [2]Department of Clinical Pharmacology, Portuguese Institute of Oncology, Porto, Portugal; and [3]Institut für Anthropologie und Humangenetik, Universität Tübingen, Tübingen, Germany

The aim of this study was to examine the prevalence of pS2 expression in gastric carcinoma and to determine its prognostic significance. We analysed pS2 protein expression in 50 gastric carcinomas and respective adjacent mucosas by immunohistochemistry and immunoradiometric assay (IRMA). pS2 was consistently expressed in superficial and foveolar epithelium of non-neoplastic mucosa and in 66.0% of the carcinomas. pS2 immunoreactivity was significantly higher in diffuse than in intestinal carcinomas, and in those cases with nodal metastases than in those without. No correlation was found between pS2 immunostaining and gender, age, staging, depth of wall penetration, venous invasion, ploidy and S-phase fraction. The mean levels of pS2 (IRMA) were significantly lower in gastric carcinomas than in non-neoplastic mucosas, and were not correlated with any of the aforementioned clinicopathological features. The survival of patients with pS2-positive tumours was not significantly different from that of patients with pS2-negative tumours. We conclude that pS2 expression, which can be used as a marker of gastric-type differentiation, is associated with gastric carcinoma of diffuse type. The lack of correlation between pS2 expression and most features of tumour aggressiveness and patients' survival precludes its use as a prognostic tool in gastric carcinoma.

Key words: gastric carcinoma, immunohistochemistry, immunoradiometric assay (IRMA), mucins, pS2, stomach, survival, trefoil peptides

Eur J Cancer, Vol. 32A, No. 9, pp. 1585–1590, 1996

INTRODUCTION

pS2 PROTEIN is a member of the trefoil peptide family whose gene was cloned from the MCF-7 breast cancer cell line by Masiakowski and colleagues [1]. In human breast cancer, pS2 is associated, though not exclusively, with oestrogen receptor expression [2–4], responsiveness to hormone therapy [5] and favourable prognosis [6, 7].

In normal human tissues, and excluding the pS2 expression in some apparently normal breast specimens [4, 8], pS2 has only been consistently detected in the superficial and foveolar epithelium of gastric mucosa [9–14].

The few published reports on pS2 immunoexpression in gastric carcinoma [10, 14–16] provide discrepant data on some of the clinicopathological parameters under analysis. Theisinger and colleagues [16] observed pS2 expression of variable intensity in every carcinoma of their series, whereas the prevalence of pS2 immunoreactivity varied from 48 to 57 per cent in other series [10, 14, 15]. In contrast to Müller and Borchard [14] who did not find any significant relationship between pS2 expression and the histological type, Theisinger and colleagues [16] found a close association between diffuse gastric carcinoma and a high percentage of strongly stained pS2 immunoreactive cells. Finally, Müller and Borchard [14] reported a significant relationship between pS2 expression and extent of tumour growth (pT stage); despite this, they did not observe any significant influence of the immunohistochemical expression of pS2 on the outcome of the patients [14].

We undertook the present immunohistochemical and immunoradiometric study of pS2 in a series of gastric carcinomas in an attempt to settle the aforementioned discrepancies. We also intended to determine whether the immunohistochemical evaluation of pS2 carries any meaningful

Correspondence to J.-C. Machado.
Revised 3 Feb. 1996; accepted 8 Feb. 1996.

prognostic information as has been observed in mammary and pulmonary adenocarcinomas [6, 17].

MATERIALS AND METHODS

Tissue material

Six cases of normal gastric biopsies obtained from dyspeptic patients without gastric carcinoma or other focal lesions at endoscopy were included in this study. From each patient, there were six biopsies available (two from the antrum, two from the incisura and two from the body/fundus). Surgical specimens from 50 gastric carcinomas consecutively resected at Hospital S. João-Medical Faculty (IPATIMUP), Porto, Portugal, were studied. The mucosa adjacent to each case of gastric carcinoma was also studied.

The tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 4 μm were cut from each block and used for routine staining with haematoxylin and eosin (H&E), periodic acid Schiff (PAS) and alcian blue pH 2.5/high iron diamine (HID/AB) and immunohistochemical stains. Frozen samples from 33 carcinomas were available for flow cytometry performed as previously described [18].

Immunohistochemistry

Monoclonal antibody (MAb) BC4 (CIS bio international, Gif-Sur-Yvette, France) was used for immunohistochemical (IHC) study of the expression of pS2 in formalin-fixed paraffin-embedded tissues. A modification of the avidin-biotin-peroxidase complex method [19] was applied. The paraffin sections (4 μm thick) were dewaxed, incubated for 30 min at 37°C in a 0.01 M HCl solution containing 0.4% pepsin and then rinsed in TRIS buffered saline (TBS), pH 7.6. The sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase, washed in TBS, and then incubated for 20 min with normal rabbit serum, at a dilution of 1:5 in TBS containing 25% bovine serum albumin (BSA). Excess normal serum was removed and replaced by the MAb BC4 diluted 1:4. After overnight incubation (18 h) at 4°C, slides were washed with TBS and the sections incubated with a 1:200 dilution of biotin-labelled secondary antibody for 30 min. After washing with TBS, sections were incubated with avidin-biotin-peroxidase complex (10 mg/ml of biotin-labelled peroxidase) for 60 min. This was followed by staining the sections for 5 min with 0.05% diaminobenzidine, freshly prepared in 0.05 M TRIS buffer, pH 7.6, containing 0.01% hydrogen peroxide. Finally, sections were counterstained with haematoxylin, dehydrated and mounted. Dilution of primary antibodies, biotin-labelled secondary antibodies, and avidin-biotin-peroxidase complex were made with TBS containing 12.5% BSA.

All series included normal gastric mucosa as positive controls. Negative controls were performed by substitution of the primary antibody with IgG1 immunoglobulins of the same subclass and concentration as the MAb.

Scoring

A case was considered to be negative (0) whenever histochemical or immunohistochemical staining was absent or present in only very few cells. A semiquantitative approach was used to score the staining of the positive cases into cases with moderate number of positive cells (+) and cases in which the majority of the neoplastic cells were immunoreactive (++), irrespective of the localisation of the positive cells and the intensity of the staining.

Immunoradiometric assay (IRMA) for pS2

Immunoradiometric assay for pS2 was performed in 30 tumours and 12 samples of non-neoplastic mucosa. From every case, a parallel sample of neoplastic or non-neoplastic tissue was obtained in order to monitor, by histological examination, if the IRMA samples were representative. Frozen tissues were homogenised at 0°C in a Polytron homogeniser. Homogenization buffer, pH 7.4, included 10 mM Tris, 1.5 nM EDTA, 1 mM ditiothreitol, 1% v/v monotioglycerol, and 10% v/v glycerol. The homogenates were centrifuged at 105 000 ***g*** at 0°C, for 70 min. The obtained supernatant fraction was used for immunoradiometric assay and for total protein quantification according to Bradford [20]. pS2 immunoradiometric assay kit ELSA-pS2 was purchased from CIS bio international (Gif-Sur-Yvette, France) and used according to the supplier's recommendations. Briefly, samples were incubated with pS2 ^{125}I-radiolabelled monoclonal antibody in pS2 monoclonal antibody coated tubes (standards and tissue supernatant fractions) at room temperature for 1 h. Unbound radiolabelled antibody was removed by washing the tubes and radioactivity was measured.

Statistical analysis

The results are expressed as a percentage or as a mean ± standard deviation. The statistical analysis of the results was performed by Pearson-chi squared test, unpaired Student's *t*-test and Mann–Whitney *U* test using Statview 4.01 software. Follow-up data was obtained in every case. The median follow-up was 17 months (range 3–63 months). Survival curves were calculated according to the Kaplan–Meier method and statistically compared using the Mantel–Cox test using BMDP statistical software package. Differences were considered to be statistically significant at values of $P<0.05$.

RESULTS

Normal mucosa

We found pS2 immunostaining in every case of normal gastric mucosa. pS2 expression was seen throughout the superficial and foveolar epithelium of antrum and body (Figure 1), as well as in mucopeptic cells of the neck. pS2 immunostaining was also seen, focally, in antrum glands but not in body glands. In the antral glands, the intensity of immunostaining was much weaker than in the superficial part of the mucosa. At the cellular level, the pattern of pS2 expression was mainly cytoplasmic (diffuse) but strong immunostaining was also seen in the perinuclear region (Golgi area), apical membrane and luminal secretions (Figure 1).

Mucosa adjacent to gastric carcinomas

The mucosa adjacent to gastric carcinomas displayed either superficial gastritis (six cases) or chronic atrophic gastritis (44 cases). The pS2 expression in foveolar and surface epithelium of non-neoplastic gastric mucosa adjacent to carcinomas was similar to that of normal mucosa.

Gastric carcinoma

Table 1 summarises the clinicopathological features of the 50 cases included in the present series and the immunohistochemical findings regarding pS2 expression. All 50 carcinomas were classified according to Laurén [21] as intestinal ($n = 28$), diffuse ($n = 18$) and unclassifiable ($n = 4$). pS2 immunostaining was observed in 33 of the 50 gastric carcinomas (66.0%),

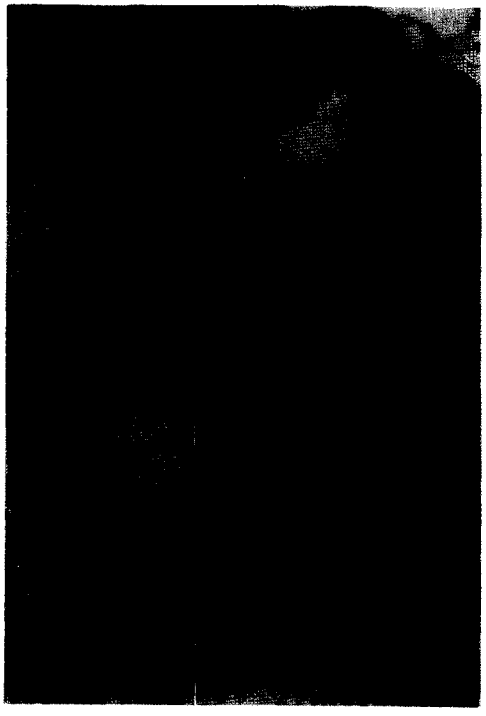

Figure 1. Normal gastric mucosa: pS2 immunoreactivity is observed throughout the superficial and foveolar epithelium of antral mucosa (original magnification × 140).

which included 16 of 18 (88.9%) diffuse carcinomas (Figure 2) and 15 of 28 (53.6%) intestinal carcinomas (Figure 3). This difference was found to be statistically significant ($P = 0.037$) (Table 1). The frequency of cases exhibiting immunoreactivity in the majority of neoplastic cells (++) was higher in diffuse (22.2%) than in intestinal (10.7%) carcinomas.

Sixteen cases, classified according to the predominant histological type as intestinal or diffuse carcinomas, displayed small

Figure 2. pS2-positive diffuse carcinoma: intense pS2 immunoreactivity is observed in almost every neoplastic cell (original magnification × 350).

Table 1. Relationship between the clinicopathological features of gastric carcinomas and pS2 expression

		pS2 IHC		
	Number of cases (%)	Negative	Positive	*P* value
Gender				
Male	34 (68.0)	12 (35.3)	22 (64.7)	
Female	16 (32.0)	5 (31.2)	11 (68.8)	NS
Age				
<40 years	9 (18.0)	2 (22.2)	7 (77.8)	
⩾40 years	41 (82.0)	15 (36.6)	26 (63.4)	NS
Stage				
I	23 (46.0)	9 (39.1)	14 (60.9)	
II	10 (20.0)	6 (60.0)	4 (40.0)	
III	16 (32.0)	2 (12.5)	14 (87.5)	
IV	1 (2.0)	0 (0)	1 (100)	NS
Histological type				
Intestinal	28 (56.0)	13 (46.4)	15 (53.6)	
Diffuse	18 (36.0)	2 (11.1)	16 (88.9)	
Unclassifiable	4 (8.0)	2 (50.0)	2 (50.0)	0.037
Depth of invasion				
Mucosa and submucosa	9 (18.0)	2 (22.2)	7 (77.8)	
Muscular and serosa	41 (82.0)	15 (36.6)	26 (63.4)	NS
Metastases to the lymph nodes				
Negative	28 (56.0)	13 (46.4)	15 (53.6)	
Positive	22 (44.0)	4 (18.2)	18 (81.8)	0.036
Venous invasion				
Negative	29 (58.0)	11 (37.9)	18 (62.1)	
Positive	21 (42.0)	6 (28.6)	15 (71.4)	NS
Ploidy ($n = 33$)				
Diploid	16 (48.5)	8 (50.0)	8 (50.0)	
Aneuploid	17 (51.5)	7 (41.2)	10 (58.8)	NS
Total	50 (100)	17 (34)	33 (66)	

NS, not significant.

Figure 3. pS2-positive intestinal carcinoma: pS2 immunoreactivity is observed in less than 50% of the neoplastic cells (original magnification × 140).

foci with a structure different from the main component of the tumour—foci of intestinal carcinoma in diffuse carcinomas ($n = 10$) and foci of diffuse carcinomas in intestinal carcinomas ($n = 6$). pS2 immunoreactivity was observed in every one of these 'mixed' carcinomas. In most cases ($n = 12$), immunoreactivity was observed in both the diffuse and the intestinal component of the tumours (Figure 4). In the remaining four cases, immunostaining was restricted to the diffuse component (Figure 5).

In the whole series we found a significant correlation between pS2 immunostaining and metastasis to lymph nodes ($P = 0.036$) (Table 1). Within each group of the different histological types, no significant correlation was found between pS2 expression and lymph node metastases (data not shown).

No significant correlations were found between pS2 immunostaining and the gender or age of patients, stage of the neoplastic disease, depth of penetration of gastric wall and venous invasion (Table 1). Similarly, no significant correlation was observed with regard to ploidy (Table 1) and S-phase fraction (SPF) ($16.8 \pm 16.5\%$ in the positive cases and $17.4 \pm 10.3\%$ in the negative cases).

The comparison of the pS2 immunoexpression with mucin histochemical expression in serial sections of the gastric carcinomas revealed that there was a close topographic overlap

Figure 4. In this 'mixed' carcinoma pS2 immunoreactivity is observed in both components of the tumour: intestinal component (left) and diffuse component (right) (original magnification × 350).

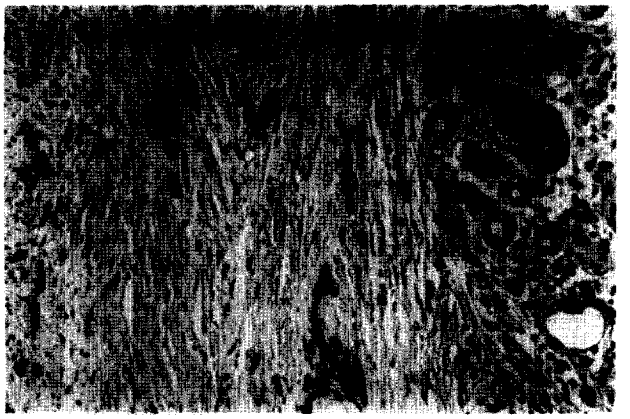

Figure 5. In this 'mixed' carcinoma pS2 immunoreactivity is restricted to one of the components of the tumour: diffuse component (left) with positive cells and intestinal component (right) with no immunoreactive cells (original magnification × 350).

between both stainings, regardless of mucin type (neutral and/or acid) and the histological type of the tumours.

The mean level of pS2 in gastric carcinomas as measured by IRMA was significantly lower ($P = 0.05$) than that of non-neoplastic mucosa (58.8 ± 42.6 and 76.5 ± 21.6, respectively). pS2 mean level detected by IRMA was higher in immunohistochemical positive cases (64.5 ± 46.9) than in negative cases (52.3 ± 37.8), but the difference did not attain the threshold of statistical significance. No significant correlation was found between the mean levels of pS2 and the different clinicopathological features of the cases, namely the histological type of the carcinomas and the presence of nodal metastases.

The comparison of the postoperative survival curves of patients with pS2-positive and patients with pS2-negative gastric carcinomas revealed no significant difference between the two groups (Figure 6).

DISCUSSION

We found pS2 expression in 66.0% of the gastric carcinomas, a frequency that is close to those reported by Luqmani and colleagues [10], Henry and colleagues [15] and Müller and Borchard [14] (57, 56 and 48%, respectively) and substantially inferior to that reported by Theisinger and colleagues [16]. Differences in the methodologies and criteria used in the five series probably account for the discrepancies.

We observed a significantly higher frequency of pS2 im-

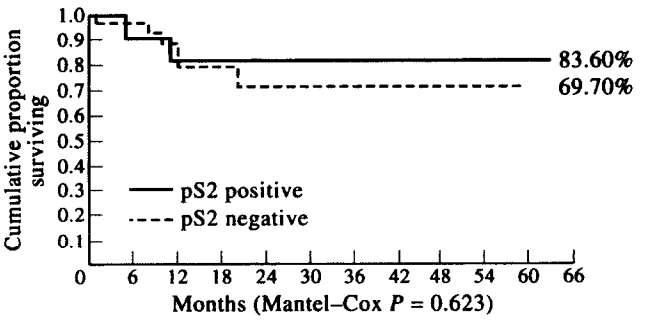

Figure 6. Survival curves of patients with pS2-positive and pS2-negative gastric carcinoma.

munoreactivity in diffuse carcinomas (88.9%) than in intestinal carcinomas (53.6%). Furthermore, we observed that the frequency of carcinomas displaying immunoreactivity in the majority of neoplastic cells was higher in diffuse than in intestinal carcinomas (22.2 and 10.7%, respectively). These findings concur with those of Theisinger and colleagues [16] and contrast with the results obtained by Müller and Borchard [14] who did not find any significant relationship between pS2 expression and the histological type of gastric carcinoma. The close relationship we found between pS2 expression and carcinomas of the diffuse type was also observed in some of the 'mixed' carcinomas of the present series, which displayed immunoreactivity in the diffuse component in contrast to the absence of immunostaining in the intestinal component.

Müller and Borchard [14] found a highly significant correlation between pS2 immunoreactivity and expression of markers of gastric differentiation, such as pepsinogen II and 2B5, which were co-expressed in most of the pS2 immunoreactive cells. If we assume, as Müller and Borchard [14] did, that pS2 immunoreactivity discloses the gastric phenotype of neoplastic cells, we may conclude that most diffuse carcinomas (88.9%) display a gastric-type differentiation, which is exhibited in the majority of the neoplastic cells in about one-fifth of the cases. These findings are in accordance with the evidence provided by ultrastructural studies, which have shown that gastric-type cells (foveolar and/or mucopeptic) are observed in the majority of diffuse carcinomas [22, 23]. Furthermore, almost half of the intestinal carcinomas of our series—those displaying pS2 immunostaining—exhibited focally (42.9% of the cases) or extensively (10.7% of the cases) signs of gastric-type differentiation. These findings are in agreement with the data of Fiocca and colleagues [24] who showed that, in their series, 55% of the cases of gastric carcinoma with glandular structure expressed pepsinogen II. Kushima and Hattori [25] searched for signs of gastric and intestinal differentiation using histochemical methods and showed that gastric-type differentiation was present—exclusively or in association with intestinal-type differentiation—in 69.8% of differentiated-type carcinomas (with glandular structure). The evidence provided by ultrastructural studies [22, 23] also demonstrates the presence of different cell types, including foveolar and mucopeptic cells (gastric-type cells), besides intestinal columnar or goblet cells (intestinal-type cells) in gastric carcinomas forming glands [22, 23].

Overall, the present and the aforementioned studies provide enough evidence to claim that gastric-type differentiation is present both in diffuse and intestinal types of gastric carcinoma, though much more often and more expressively in the former than in the latter. The prominence of cellular differentiation of gastric-type in diffuse carcinomas suggests that this is the type of carcinoma which is more closely linked to the gastric mucosa both histogenetically and from a differentiation standpoint, whereas the so-called intestinal carcinomas encompass gastric-type adenocarcinomas, carcinomas with intestinal-type differentiation and tumours with dual differentiation. The latter finding reinforces our previous contention that gastric carcinomas with gland formation should be designated as 'glandular' carcinomas instead of 'intestinal' carcinomas [26], in order to avoid the mixture of structural and cell differentiation concepts.

We found a significant correlation between pS2 expression and lymph node metastases. This finding is a side-effect related to the histological type since, in our series, most of the cases with lymph node metastases were diffuse carcinomas (68.2%) which were also those expressing more often pS2 immunoreactivity. Within each histological type, no significant correlation was found between pS2 immunoreactivity and nodal metastases. We found no other significant correlation of pS2 immunoreactivity and the clinicopathological parameters under analysis. These results contrast with those of Müller and Borchard [14] who found, in a series of 120 gastric carcinomas, a significant relationship between pS2 expression and extent of tumour growth (pT stage).

There was a lack of agreement between the results of immunohistochemical study of pS2 expression and those obtained by IRMA, except for the demonstration of higher levels of pS2 in the non-neoplastic mucosas compared with carcinomas. By IRMA, we did not find any significant correlation between pS2 levels and the different clinicopathological parameters, namely regarding the histological type of the tumours. The discrepancy between immunohistochemistry and IRMA may reflect both the effect of tumoural heterogeneity regarding pS2 expression and the effect of stromal contamination. The latter possibility provides a putative explanation for the finding of similar pS2 levels, detected by IRMA, in diffuse and intestinal carcinomas, in contrast to immunohistochemical results which showed that pS2 expression was significantly higher in diffuse than in intestinal carcinomas, since the abundance of stromal, non-neoplastic tissue, is higher in the former than in the latter. It remains to be seen if the correction of cytosolic values for percentage of epithelial cells in the tumour samples will provide less discrepant results with the two methods, as recently suggested by Willemse and colleagues [27].

At variance with breast cancer, pS2 expression does not correlate in gastric carcinoma with the expression of oestrogen receptors (ER). In 39 of 50 cases included in the present series, we found no expression of female sex hormone receptors (ER and progesterone receptors) in the normal mucosa of the stomach nor in the cells of the carcinomas [28].

In gastric carcinomas, we found a good correlation between the amount and topographic distribution of pS2 positive and mucus-secreting cells. Our preliminary studies (data not shown) on the immunoexpression of MUC-5, in serial sections, indicate a topographic overlap between pS2 positive and MUC-5 positive cells. These findings support the concept that there is a co-expression of pS2 and mucins, as previously suggested by Wright and colleagues [29].

We found no statistically significant difference in the survival of patients with pS2-positive and pS2-negative tumours. Despite the too short follow-up of most cases, our results are in keeping with those obtained by Müller and Borchard [14] in their series of 120 gastric carcinomas, and contrast with the results observed in breast cancer [6] and lung cancer [17], in which pS2 immunoreactivity is associated with a more favourable or a less favourable prognosis, respectively.

In conclusion, our results show that in gastric carcinomas, pS2 expression reflects gastric-type differentiation and is significantly associated to the diffuse type of gastric carcinoma. pS2 expression is neither associated with features of tumour aggressiveness nor influences the survival of patients with gastric carcinoma, thus being of no value for prognostic purposes.

1. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P. Cloning of cDNA sequences of hormone-regulated

genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Res* 1982, **10**, 7895–7903.
2. Rio MC, Bellocq JP, Gairard B, *et al.* Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene ERBB2. *Proc Natl Acad Sci USA* 1987, **84**, 9243–9247.
3. Skilton RA, Luqmani YA, McClelland RA, Coombes RC. Characterisation of a messenger RNA selectively expressed in human breast cancer. *Br J Cancer* 1989, **60**, 168–175.
4. Pallud C, Le Doussal V, Pichon MF, Prud'homme JF, Hacene K, Milgrom E. Immunohistochemistry of pS2 in normal human breast and in various histological forms of breast tumours. *Histopathology* 1993, **23**, 249–256.
5. Henry JA, Nicholson S, Hennessy C, Lennard TW, May FE, Westley BR. Expression of the oestrogen regulated pNR-2 mRNA in human breast cancer: relation to oestrogen receptor mRNA levels and response to tamoxifen therapy. *Br J Cancer* 1990, **61**, 31–38.
6. Foekens JA, Rio MC, Seguin P, *et al.* Prediction of relapse and survival in breast cancer patients by pS2 protein status. *Cancer Res* 1990, **50**, 3832–3837.
7. Cappelletti V, Coradini D, Scanziani E, Benini E, Silvestrini R, Di Fronzo G. Prognostic relevance of pS2 status in association with steroid receptor status and proliferative activity in node-negative breast cancer. *Eur J Cancer* 1992, **28A**, 1315–1318.
8. Hähnel E, Robbins P, Hähnel R. Expression of the pS2 gene in normal breast tissue. *Breast Cancer Res Treat* 1993, **28**, 295–297.
9. Rio MC, Bellocq JP, Daniel JY, *et al.* Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. *Science* 1988, **241**, 705–708.
10. Luqmani Y, Bennett C, Paterson I, *et al.* Expression of the pS2 gene in normal, benign and neoplastic human stomach. *Int J Cancer* 1989, **44**, 806–812.
11. Wright NA, Poulsom R, Stamp GW, *et al.* Epidermal growth factor (EGF/URO) induces expression of regulatory peptides in damaged human gastrointestinal tissues. *J Pathol* 1990, **162**, 279–284.
12. Piggott NH, Henry JA, May FE, Westley BR. Antipeptide antibodies against the pNR-2 oestrogen-regulated protein of human breast cancer cells and detection of pNR-2 expression in normal tissues by immunohistochemistry. *J Pathol* 1991, **163**, 95–104.
13. Hanby AM, Poulsom R, Singh S, Elia G, Jeffery RE, Wright NA. Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. *Gastroenterology* 1993, **105**, 1110–1116.
14. Müller W, Borchard F. pS2 protein in gastric carcinoma and normal gastric mucosa: association with clinicopathological parameters and patient survival. *J Pathol* 1993, **171**, 263–269.
15. Henry JA, Bennett MK, Piggott NH, Levett DL, May FE, Westley BR. Expression of the pNR-2/pS2 protein in diverse human epithelial tumours. *Br J Cancer* 1991, **64**, 677–682.
16. Theisinger B, Welter C, Seitz G, *et al.* Expression of the breast cancer associated gene pS2 and the pancreatic spasmolytic polypeptide gene (hSP) in diffuse type of stomach carcinoma. *Eur J Cancer* 1991, **27A**, 770–773.
17. Higashiyama M, Doi O, Kodama K, *et al.* Prognostic significance of pS2 protein expression in pulmonary adenocarcinoma. *Eur J Cancer* 1994, **30A**, 792–797.
18. David L, Sansonetty F, Soares P, *et al.* DNA content of gastric lesions with particular emphasis on gastric carcinoma. A study of 71 consecutive cases using flow cytometry. *Surg Pathol* 1994, **5**, 211–224.
19. Hsu SM, Raine L, Fanger H. A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am J Clin Pathol* 1981, **75**, 734–738.
20. Bradford MM. A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein dye binding. *Anal Biochem* 1976, **72**, 248–254.
21. Laurén P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965, **64**, 31–49.
22. Fiocca R, Villani L, Tenti P, *et al.* Characterization of four main cell types in gastric cancer: foveolar, mucopeptic, intestinal columnar and goblet cells. An histopathologic, histochemical and ultrastructural study of 'early' and 'advanced' tumors. *Pathol Res Pract* 1987, **182**, 308–325.
23. Carneiro F, Moutinho-Ribeiro M, David L, *et al.* Signet ring cell carcinoma of the stomach: a morphometric, ultrastructural and DNA cytometric study. *Ultrastruct Pathol* 1992, **16**, 603–614.
24. Fiocca R, Cornaggia M, Villani L, Capella C, Solcia E, Samloff IM. Expression of pepsinogen II in gastric cancer. Its relationship to local invasion and lymph node metastases. *Cancer* 1988, **61**, 956–962.
25. Kushima R, Hattori T. Histogenesis and characteristics of gastric-type adenocarcinomas in the stomach. *J Cancer Res Clin Oncol* 1993, **120**, 103–111.
26. Carneiro F, Seixas M, Sobrinho-Simões M. New elements for an updated classification of the carcinomas of the stomach. *Pathol Res Pract* 1995, **191**, 571–584.
27. Willemse F, Nap M, Eggink HF, Foekens JA, Henzenlogmans SC, Vanputten WLJ. Quantification of relative area of pS2 immunohistochemical staining and epithelial percentage in breast carcinomas: the effect of the latter on the interpretation of a cytosolic pS2 assay. *Mod Pathol* 1995, **8**, 521–525.
28. Machado JC, Carneiro F, Gärtner F, Ribeiro P, Sobrinho-Simões M. Female sex hormone receptors are not involved in gastric carcinogenesis. A biochemical and immunohistochemical study. *Eur J Cancer Prev* 1994, **3**, (Suppl. 2), 31–37.
29. Wright NA, Poulsom R, Stamp G, *et al.* Trefoil peptide gene expression in gastrointestinal epithelial cells in inflammatory bowel disease. *Gastroenterology* 1993, **104**, 12–20.

Acknowledgements—The study was supported by JNICT (grant PECS/C/SAV/220/95), DFG (grant BL 166/11-1) and DAAD/CRUP (travel support).