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Prognostic Significance of pS2 Protein Expression in Pulmonary Adenocarcinoma

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In the present study, pS2 protein expression in pulmonary adenocarcinoma was investigated on paraffin-embedded sections obtained from 170 patients. 28 (16%) patients showed varying degrees of pS2 protein expression in the cytoplasm of tumour cells, as detected by immunohistochemical staining with anti-pS2 protein antibody. There was a significant association between pS2 protein expression and larger tumour size, and the acinar or bronchiolo-alveolar subtype. However, no significant correlations between pS2 protein status and the other clinicopathological factors, i.e. T-factor, N-factor, stage and histological differentiation, were shown. In contrast to breast cancer, patients with pS2-positive pulmonary adenocarcinomas had a significantly worse prognosis than those with pS2-negative pulmonary adenocarcinomas; this was true for stage I patients, as well as for all patients. Multivariate analysis showed that pS2 protein expression was a discriminating variable in overall survival. These findings suggest that pS2 protein status is a possible prognostic indicator in pulmonary adenocarcinoma.

Key words: pulmonary adenocarcinoma, pS2 protein, immunohistochemistry, prognosis
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INTRODUCTION

THE pS2 GENE was originally isolated by Chambon and co-workers [1] as a gene whose transcript, including mRNA of about 600 nucleotides [2], has been shown to be directly regulated by oestrogen in the human breast cancer cell line, MCF-7. The 5′ flanking sequence of the gene includes an oestrogen-inducible promoter [3]. The pS2 gene contains a single open reading frame encoding an 84 amino acid long secretory protein [2], whose function is still unknown. Recent studies, using a cDNA probe for pS2 mRNA and specific antibodies against the pS2 protein, have shown that pS2 is predominantly expressed in oestrogen receptor-positive breast tumours [4–10]. These findings in breast

cancer indicate that pS2 status, like oestrogen receptor status, is a favorable indicator for assessing response to oestrogen hormonal therapy [6, 7, 9, 10]. Furthermore, pS2 appears to be an important prognostic variable; patients with pS2-negative breast cancer have been shown to be at higher risk than those with pS2-positive breast cancer [6, 7, 9,10].

Various immunohistochemical analyses have revealed that pS2 protein is also expressed in several other malignancies, i.e. in colorectal [11, 12], pancreatic [11, 13], gastric [11, 14, 15] and gynaecological [11, 16] cancers, and interestingly, this expression seems to be independent of oestrogen receptor status in almost all cases. In lung cancer, a preliminary study showed that the pS2 protein is expressed in pulmonary adenocarcinoma [11, 12]. However, in contrast to breast cancer, neither the mechanism underlying this protein expression nor its clinical significance have been determined.

Accordingly, to determine the relationship between pS2 protein expression and clinicopathological characteristics, we analysed pulmonary adenocarcinoma specimens for pS2 protein expression, using immunohistochemical techniques employing an anti-pS2 protein monoclonal antibody, and also evaluated the prognostic value of this protein expression.

MATERIALS AND METHODS

Source of tissues

We prepared formalin-fixed and paraffin-embedded tissue blocks from 170 surgically resected specimens of pulmonary adenocarcinoma at our institute, which were well-preserved for immunohistochemistry. Of the 170 patients who were followed-up for a median time of 42.5 months (range 3.0–125.0), 107 were male and 63 were female; their ages ranged from 19 to 79 years (mean 61.4). According to the international TNM staging system [17], 83 were in pathological stage I (p-stage I), 17 were in pathological stage II (p-stage II), 61 were in pathological stage IIIA (p-stage IIIA), 4 were in pathological stage IIIB (p-stage IIIB), and 5 were in pathological stage IV (p-stage IV). With regard to histological differentiation, 59 were well differentiated, 74 were moderately differentiated and 37 were poorly differentiated. 12 patients underwent non-curative surgery; this was due to pulmonary metastasis within the resected specimen in 5, intrathoracic dissemination in 4 and residues of surgical margin in 3 patients.

Immunohistochemistry

Immunohistochemistry was performed using the avidin–biotin complex peroxidase method, as described previously [18]. Briefly, 5-μm sections, including the maximum cut-surface specimens of the tumour tissues, were cut from paraffin blocks and allowed to air dry. The slides were deparaffinised in xylene and absolute alcohol, and treated with 0.3% hydrogen peroxidase–methanol to remove endogenous peroxidase activity. After washing, the slides were treated with 2% normal horse serum to reduce background staining, and incubated overnight with the primary antibody (dilution of 1:2) at 4°C in a moister chamber. The primary monoclonal anti-pS2 protein antibody, designated BC4 [6], was purchased from CIS Bio International Corp. (France). This antibody was produced against the pS2 protein isolated from the culture medium of MCF-7 human breast cancer cells [6]. After washing, the slides were incubated with the secondary antibody for 30 min (biotinylated anti-mouse IgG; dilution of 1:250, Vector Laboratories, California, U.S.A.), washed and then treated with the avidin–biotin–peroxidase reagent (Vector Elite) for 30 min. The antigen–antibody complex was visualised using a 0.05% solution of diaminobenzidine tetrahydrochloride in phosphate-buffered saline (PBS). The slides were counterstained with Mayer's haematoxylin, dehydrated and mounted with Permount.

Analysis

pS2 protein staining in the tissues of pulmonary adenocarcinomas was assessed by light microscopy by two observers. Staining results were evaluated semi-quantitatively, taking into account the percentage of cytoplasmic pS2 protein-positive tumour cells in the maximum cut-surface specimen sections. More than 1% of tumour cells showing immunoreactivity for pS2 protein in the cytoplasm were judged pS2 protein-positive (Figure 1), and the others were regarded as negative. In several patients' tissues, membranous staining in the luminal surface, not the cytoplasm, of the tumour cells was focally observed, but such cases were included as pS2 protein negatives (See Results and Discussion).

The χ^2 test was applied for statistical analysis. Patients' survival data were used to determine the possible correlation between pS2 protein expression and cancer-associated death. Survival curves were constructed using Kaplan–Meier analysis. The statistical significance of these data was analysed by the generalised Wilcoxon test. Variables related to survival were analysed by Cox's proportional hazards regression model with SAS software (Statistical Analysis System Institute, Cary, North Caroline, U.S.A.) [19].

RESULTS

Expression of pS2 protein in non-cancerous pulmonary tissue

With immunohistochemical staining using anti-pS2 protein antibody (BC4), bronchial glands, i.e. mucus glands and their ducts, showed strong immunoreactivity in the cytoplasm, while serous glands contained no pS2 protein (data not shown). Neither bronchial epithelium nor alveolar cells expressed pS2

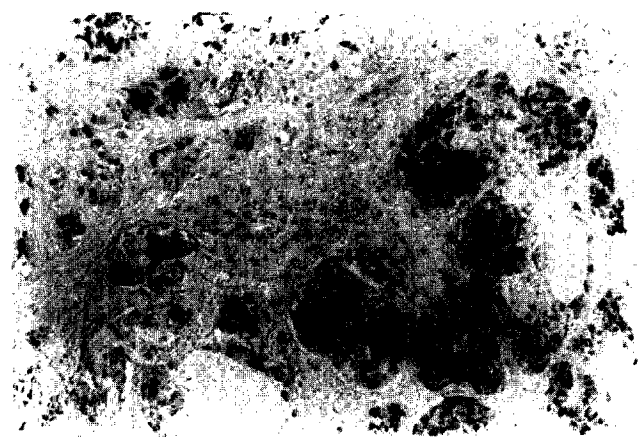

Figure 1. pS2 protein expression in pulmonary adenocarcinoma. Immunostaining using anti-pS2 protein antibody (haematoxylin counterstaining). The majority of pulmonary adenocarcinoma cells contain pS2 protein in the cytoplasm. Original magnification × 25.

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protein in the cytoplasm, but weak staining in the membranous portion of such cells was observed focally in some of the tested specimens.

Expression of pS2 protein in pulmonary adenocarcinoma

Of 170 patients, 28 (16%) expressed pS2 protein in the cytoplasm of the tumour cells, admixed with pS2 protein-positive and negative tumour cells in varying degrees within the tumour tissues (Figure 1). As described in Materials and Methods, patients with focal membranous staining were classified as pS2 protein negative. Table 1 shows the relationship between pS2 protein expression and clinicopathological factors. In this series, no significant differences between pS2 protein expression and sex, age, stage, T-factor, N-factor, pulmonary metastasis, histological differentiation and curability were found between the pS2 protein-positive and -negative groups. However, there was a significant association between pS2 protein expression and tumour size, with larger tumours showing pS2 protein expression more frequently than smaller tumours ($P = 0.0198$). With regard to subtype, the acinar or bronchiolo-alveolar subtype of pulmonary adenocarcinoma showed significantly higher frequency of pS2 protein expression than did the papillary subtype ($P < 0.0001$).

Survival analysis

Postoperative overall survival curves for these two groups are shown in Figure 2a. The average 5-year survival rates were 36% in pS2 protein-positive patients and 60% in protein-negative patients ($P = 0.0195$).

Figure 2b shows that the survival curves for 83 p-stage I patients according to the pattern of pS2 protein expression. The average 5-year survival rates of each group were 53 and 84%, respectively ($P = 0.0349$). Patients with pS2 protein expression showed a significantly poorer survival than those with no pS2 protein expression.

To determine the independent predictive value of pS2 protein expression status for postoperative overall survival, we performed a multivariate analysis on data from 159 potentially cura-

Table 1. pS2 protein expression in pulmonary adenocarcinoma

Number	Negative ($n = 142$)	Positive ($n = 28$)	P value*
Sex			
Male	90	17	
Female	52	11	NS
Age (years)			
< 41	4	1	
41-60	56	9	
≥ 61	82	18	NS
Mean	61.2	62.6	
p-stage†			
I	65	18	
II + III A + III B + IV	77	10	NS
II	16	1	
III A + III B	58	7	
IV	3	2	
T-factor†			
T1	74	9	
T2,3,4	68	19	NS
N-factor†			
N0	79	19	
N1,2	63	9	NS
Pulmonary metastasis			
−	139	26	
+	3	2	NS
Tumour size (mm)			
< 31	82	9	
31–60	51	14	
≥ 61	9	5	0.0198‡
Differentiation			
Well	49	10	
Moderate	60	14	
Poor	33	4	NS
Subtype			
Papillary	99	11	
Acinar	42	11	
Bronchiolo-alveolar	1	6	< 0.0001‡
Curability			
Curative	133	26	
Non-curative	9	2	NS

* χ^2 test. † International TNM staging system for lung cancer by Mountain [17]. ‡Significant. NS, non-significant.

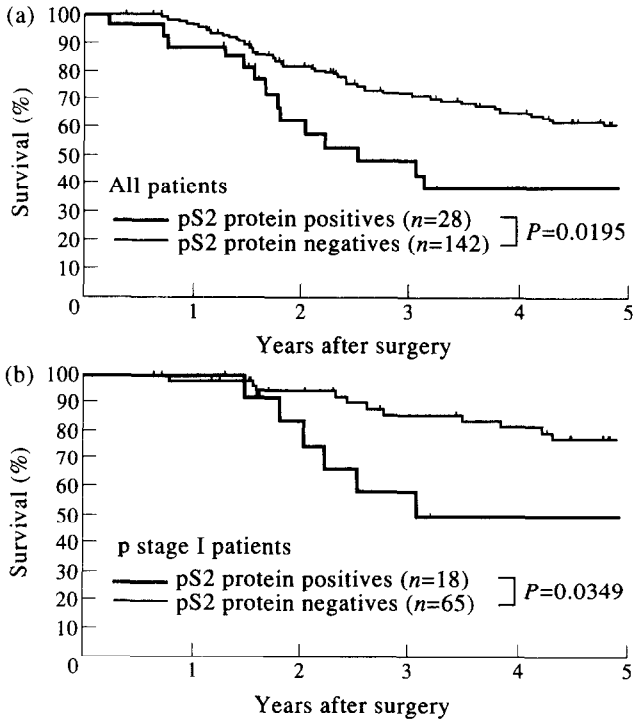

Figure 2. Postoperative overall survival curves for two groups, pS2 protein-positive and -negative patients.(a) All 170 patients. pS2 protein-positive patients have a significantly worse prognosis than pS2 protein-negative patients ($P = 0.0195$). (b) p-stage I patients. pS2 protein-positive patients have also significantly worse prognosis than pS2 protein-negatives ($P = 0.0349$).

tively resected patients (Table 2). pS2 protein expression status was remarkably discriminant for overall survival ($\beta = 0.496$, S.E. $= 0.153$, $\chi^2 = 10.528$, $P = 0.0014$) and p-stage (p-stage I versus p-stage II, IIIA, IIIB, $\beta = 0.902$, S.E. $= 0.373$, $\chi^2 = 5.848$, $P = 0.0167$), this being one of the most independent predicting variables.

DISCUSSION

Following the observations that pS2 protein production is stimulated by oestrogen in the MCF-7 breast cancer cell line [1–3, 5], it has generally been considered that pS2 protein production is regulated by the oestrogen–oestrogen receptor system. However, normal gastric mucosa [8, 14, 20] and various types of cancers [11–15] have been shown to express pS2 protein, apparently irrespective of oestrogen receptor status. In the pulmonary adenocarcinomas examined in this study, pS2 protein was demonstrated in 16% of a series of 170 pulmonary adenocarcinomas. We failed to find oestrogen receptor expression in several pS2 protein-positive cases on immunohistochemical examination [21] with the ERICA kit (data not shown), indicating that pS2 protein expression in this tumour may also be independent of the oestrogen–oestrogen receptor system. In this respect, it has been reported that a multifunctional 5′ enhancer element in the promoter region of the pS2 gene is responsive to a variety of factors, e.g. epidermal growth factor (EGF), phorbol esters and oncoproteins of c-*jun* and Ha-*ras* [22]. In fact, it has been shown that pS2 protein expression is induced in MCF-7 breast cancer cells by phorbol esters or EGF or insulin-like growth factor-I (IGF-I) [5]. These findings raise the possibility that pS2 protein production may be regulated by various biochemical factors in oestrogen receptor unrelated tumours such as pulmonary adenocarcinoma.

Several patients' tumours showed only membranous staining in luminal areas of the tumour cells (data not shown). Such a membranous staining pattern was also observed in some non-cancerous epithelia of the lung. This staining pattern has been previously described even in breast cancer by Dookeran and colleagues [23]. In the present study, these patients were regarded as negatives because the membranous staining pattern showed a weak intensity, and its distribution was limited within the tumour tissues, while cytoplasmic expression of the pS2 protein, as shown in pS2 protein-positive pulmonary adenocarcinomas, was reliable. However, since its clinicopathological significance is unclear, further study on this membranous staining may be required in the future.

Compared with papillary adenocarcinomas, the acinar or bronchiolo-alveolar type adenocarcinomas showed significantly more frequent tumour expression of pS2 protein, particularly mucus-producing adenocarcinomas, e.g. bronchial gland or goblet cell type. Considering that pS2 protein is distributed in the mucus cells of bronchial glands, it is of interest with respect to tumorigenesis and differentiation, that the bronchial gland type of adenocarcinomas frequently expressed pS2 protein. Luqmani and co-workers [12] also showed pS2 protein in all cases of bronchiolo-alveolar adenocarcinoma, although the number of cases in their study was small. Similar observations of a high frequency of pS2 protein in mucinous tumour cells have also been reported in gynaecological [11, 16] and gastric [11, 14, 15] cancers. Nevertheless, in the present study, it was observed that several cases of non-mucus-producing type adenocarcinomas also expressed a large quantity of pS2 protein, so that pS2 protein cannot be regarded as a marker of glandular or mucinous differentiation.

pS2 protein expression in pulmonary adenocarcinoma correlated with tumour size, and it was shown that patients with the pS2 protein-positive pulmonary adenocarcinomas had a significantly worse prognosis than those who were pS2 protein-negative. pS2 protein shares immunological properties with EGF [25], and is very similar in sequence and structure to porcine pancreatic spasmolytic polypeptide (PSP) and human

Table 2. Multivariate analysis of Cox's proportional hazards model in 159 curatively resected patients

Variable	Multivariate analysis β (coefficient)	S.E.	χ^2	*P* value
pS2 protein status	0.496	0.153	10.528	0.0014
p-stage*	0.902	0.373	5.848	0.0167

*International TNM staging system for lung cancer by Mountain [17].

spasmolytic polypeptide (hSP) [20, 25], with for example, trefoil disulphide loop structures being a common feature in these peptides [25]. PSP, which was originally isolated from a purification fraction of porcine insulin, and which is secreted into the pancreatic fluid to inhibit intestinal motility and gastric acid secretion [26], was demonstrated to have growth stimulating properties in a human colon carcinoma cell line and in MCF-7 breast cancer cells [27]. These peptides, including pS2 protein and hSP, are coexpressed in the proliferative zone of mucosal ulceration in the digestive tract [28, 29], suggesting that they may participate in tissue healing via their effects on cell proliferation, as does EGF [28]. Together, these peptides have, therefore, been proposed to represent members of a new family of growth factors [2, 25, 27], although the pS2 protein has not been directly demonstrated to have a growth-stimulating function in tumour cells. This evidence, taken together with our results, leads us to speculate that pS2 protein in pulmonary adenocarcinoma may possibly play a role in tumour development, closely associated with prognosis in this disease.

Our findings regarding pS2 protein status in pulmonary adenocarcinoma, namely, that patients with increased pS2 protein expression have a worse prognosis, appear to be contrary to the findings in breast cancer, in which patients with pS2-negative breast cancers have a worse prognosis than pS2-positive patients [6, 7, 9]. In this respect, even in breast cancer, Cappelletti and co-workers [9] reported that pS2 protein status in node-negative patients might lack prognostic value, since they found that pS2-positive expression in oestrogen receptor-negative patients predicted a rather shorter relapse-free survival. By immunhistochemical analysis in breast cancer, Luqmani and co-workers [30] have recently emphasised that, since there was no correlation between pS2 protein and response to hormonal therapy, irrespective of oestrogen-receptor status, the clinical significance of pS2 protein might require re-evaluation. Thus, since biological behaviour may be greatly modified by various chemo-hormonal adjuvant therapies in breast cancer, whether pS2 protein-positive breast cancer has a better prognosis remains questionable.

We believe that this present study may be the first to have determined the clinicopathological significance of pS2 protein status, in particular, the correlation between pS2 protein and clinical prognosis, in cancer other than of the breast. Although our findings are preliminary in nature, they do suggest that pS2 protein expression in pulmonary adenocarcinoma may be a suitable prognostic indicator. It also seems possible that the clinicopathological significance of pS2 protein in malignancies varies in different tissues, e.g. depending on oestrogen receptor status. Further studies are thus needed to clarify not only the biological function of pS2 protein, but also its significance in each type of malignant tissue.

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A Randomised Trial of Vindesine plus Interferon-α2b Compared With Interferon-α2b or Vindesine Alone in the Treatment of Advanced Malignant Melanoma

D. A. Vorobiof and W. R. Bezwoda

60 patients were entered into a randomised study comparing vindesine (3 mg/m²/week) plus interferon-α2b (6 U/m² 3 times per week) to vindesine alone or to interferon alone for the treatment of metastatic malignant melanoma. Patients receiving the combination therapy arm (schedule A; vindesine plus interferon-α2b) showed a complete and partial response rate of 8/20 (40%) which was significantly higher ($P < 0.05$) than that achieved with either single-agent treatment schedule. In addition, patients receiving the combined treatment schedule had a significantly prolonged survival (median 19 months) when compared to a median of 10 months for interferon alone and 5 months for vindesine alone. The combination was generally well tolerated with only additive toxicity. It is concluded that combination treatment regimens utilising interferons together with chemotherapeutic agents deserve further study in the treatment of metastatic malignant melanoma.

Key words: malignant melanoma, treatment, interferon-α2b, vindesine
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INTRODUCTION

MALIGNANT MELANOMA is one of the most common malignancies in young adults. The first published report of a patient with metastatic malignant melanoma was by Hunter in 1787 [1]. The incidence of melanoma has risen rapidly in white populations during the last 50 years. Well-known risk factors are sun exposure (particularly heavy intermittent, rather than continuous exposure), the number and type of pigmented naevi and a family history of melanoma [2].

It is estimated that 70 000 new cases of malignant melanoma occur each year worldwide. In certain countries, such as Australia, melanoma is one of the five most common cancers.

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The clinical course of malignant melanoma varies widely, from a high cure rate for superficial, localised disease to an invariably fatal condition when metastases have occurred. Patients with advanced malignant melanoma remain a challenge to medical oncologists. While chemotherapy remains the mainstay of treatment for disseminated disease, the introduction of biological response modifiers has added new treatment modalities to the therapeutic armamentarium.

Vindesine, a vinca alkaloid derivative, given either as a single agent or in combination, has been shown to produce a response rate similar to that reported with other cytostatics, such as DTIC and the nitrosoureas in advanced malignant melanoma [3–5]. Interferon (IFN)-α has also been shown to be an effective treatment, with response rates of up to 29% in published reports [6]. The combination of cytostatics and IFNs is a new therapeutic approach in the treatment of advanced malignant melanoma. IFN-α has been combined with DTIC [7–11], vinblastine [12–13], cisplatin [14–16], vindesine [17] and multidrug cytos-