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# Immunoperoxidase Staining for Carcinoembryonic Antigen in Small Cell Carcinoma of the Lung

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**Abstract**—*Diagnostic pretreatment biopsies in 53 patients with small cell carcinoma of the lung (SCCL) were examined retrospectively for presence of carcinoembryonic antigen (CEA). The unlabelled antibody-enzyme (PAP) technique was utilized. All patients had been treated with intensive chemotherapy. The patients were divided into 3 groups according to the immunoperoxidase staining reactions of the biopsies. Thirteen (24.5%) patients had biopsies that were strongly positive for CEA while 22 (41.5%) had slightly positive and 18 (34%) had negative biopsies. The patients with biopsies strongly positive for CEA had a significantly longer survival as compared with patients with negative staining results ($P < 0.05$). No significant correlation was observed between immunoperoxidase staining for CEA and morphological subtypes of SCCL according to the WHO 1977 classification.*

## INTRODUCTION

THE ONCOFETAL glycoprotein carcinoembryonic antigen (CEA) was originally described by Gold and Freedman in 1965 [1]. In 1978, Newmann *et al.* reported that an antiserum against viable cultured oat cell carcinoma cells contained high titres of anti-CEA antibodies [2]. Using a triple-layer immunoperoxidase method in a series of carcinomas of the lung, Fenoglio *et al.* demonstrated CEA in all cases of "undifferentiated carcinoma" (number not stated) [3]. Sun *et al.* examined 16 anaplastic small cell carcinomas of the lung using the peroxidase–antiperoxidase technique. They found that most cases were negative for CEA (number not stated) [4]. Pascal *et al.* studied 8 cases of "undifferentiated carcinomas" of the lung using the three-layer immunoperoxidase technique. They found positive staining for CEA in 4 cases [5]. Finally, Hill *et al.* found positive staining for CEA in all 3 cases of oat cell carcinomas of the lung using the peroxidase–antiperoxidase technique [6].

The purpose of the present study was to examine pretreatment biopsies in patients with SCCL for presence of CEA using the unlabelled antibody-enzyme (PAP) method of Sternberger [7]. The staining results were then correlated to survival after intensive chemotherapy and to the morphological subtypes of SCCL according to the WHO 1977 classification [8].

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## MATERIALS AND METHODS

### *Tissues*

The material consisted of pretreatment diagnostic biopsies from patients with SCCL, on whom autopsies had been performed consecutively at the Finsen Institute from January 1977 to June 1979. All patients had been initially staged as having either regional disease (i.e. no demonstrable disease outside one hemithorax, including regional and bilateral supraclavicular lymph nodes) or extensive disease [9]. Patients who for different reasons did not enter chemotherapeutic protocol were excluded from further study. Also excluded were patients who died within 30 days of the start of treatment and thus did not receive one cycle of chemotherapy.

### *Fixation and preparation*

The histologic material had been fixed in 10% buffered formalin and paraffin-embedded. The bioptic procedures had been performed in 9 different hospitals and it was therefore not possible to determine the exact fixation times.

*Antisera*

Specific rabbit antiserum against human CEA (DAKO, Denmark, Code no. A115, lot 038, protein concentration 7.6 g/l) was absorbed with a freeze-dried, dialyzed perchloric acid extraction of human spleen. The specificity of the antiserum was tested in our laboratory by absence of staining after absorption with antigen in 2 cases of colonic adenocarcinoma and 4 cases of strongly positive SCCL. Forty $\mu$g of antigen was needed to absorb 100 $\mu$l of antiserum. The working dilution of antiserum was found by titration to be 1/200. Swine anti-rabbit immunoglobulin (DAKO, Denmark, code no. 21-090) was used in dilution 1/20. Peroxidase rabbit anti-peroxidase (PAP) (DAKO, Denmark, code no. Z113) was used in dilution 1/40. For control staining, the immunoglobulin fractions from non-immunized rabbits (DAKO, Denmark, code no X903, protein concentration 20 g/l) was used in dilution 1/500, corresponding to the protein concentration of the specific antiserum. All antisera were diluted in Tris buffer, pH 7.6, containing 10% normal swine serum.

*Staining procedure*

Five $\mu$m-thick sections were cut from each block. One section was stained for CEA according to the unlabelled antibody-enzyme (PAP) technique of Sternberger [7], with pronase pretreatment according to Denk *et al.* [10]. Briefly, the sections were de-waxed and treated with a 0.1% pronase solution (protease type 7, Sigma 5255) at 37°C for 15 min and then rinsed in ice-cold Tris buffer. Endogenous peroxidase was blocked by immersion of sections in 1% $H_2O_2$ in ethanol for 20 min. The sections were then treated with normal swine serum for 5 min in order to reduce non-specific background staining. The sections were then incubated consecutively with rabbit antiserum to CEA, swine anti-rabbit IgG and PAP, with washes in Tris buffer between each stage. 3-Amino-9-ethylcarbazole was used as chromogen to localize peroxidase. Finally, the sections were counterstained with Mayer's haemalum, rinsed in tap water and mounted in Aquamount®. All procedures were carried out at room temperature except where otherwise stated.

A second section from each block was subjected to the same procedure except that the rabbit antiserum to CEA was substituted with the immunoglobulin fraction from non-immunized rabbits. A third section was stained routinely with haematoxylin and eosin (HE).

*Microscopic evaluation*

The immunoperoxidase-stained sections were examined by one of the authors (MS) without knowledge of patient survival or the results of the morphologic subtyping. The results were graded as follows: "strongly positive" when more than 50% of tumour cells were stained; "slightly positive" when less than 50% of tumour cells were stained; and "negative" when no staining of tumour cells was observed. Intensity of staining in individual tumour cells was not assessed. The corresponding HE-stained sections were examined by another author (FH) and classified according to the WHO 1977 classification [8]. After the microscopic evaluations were completed, the results of the immunoperoxidase staining for CEA were compared with patient survival and morphological subtyping.

*Statistical analysis*

The Mann–Whitney rank sum test was used [11].

## RESULTS

Seventy-one patients were available for study. Five patients had died within 30 days of the start of treatment and 4 patients had not entered chemotherapeutic protocols. in 2 patients the primary diagnosis had been made by cytological examination of sputum alone. In 4 patients the biopsy material was unobtainable and in one patient no remaining tumour tissue was found in the biopsy material. Reclassification excluded one patient with a large cell carcinoma and one patient with malignant carcinoid. The pretreatment biopsy material thereafter consisted of 29 bronchial biopsies and 24 biopsies from metastases. Twenty-four patients had initial regional disease and 29 had extensive disease. The median age at the start of treatment was 59 years (range, 38–71 years). The male:female ratio was 8:3. The median surivial after start of treatment in the whole group was 287 days (range, 35–763 days), in the group of patients with initial regional disease 331 days (range, 58–763 days) and in the group with initial extensive disease 247 days (range, 35–723 days).

*Immunoperoxidase staining*

Tumour cells positive in the immunoperoxidase stain for CEA displayed a diffuse reddish-brown intracytoplasmatic stain which was often accentuated at the periphery of the cell (Fig. 1). No such staining was seen in

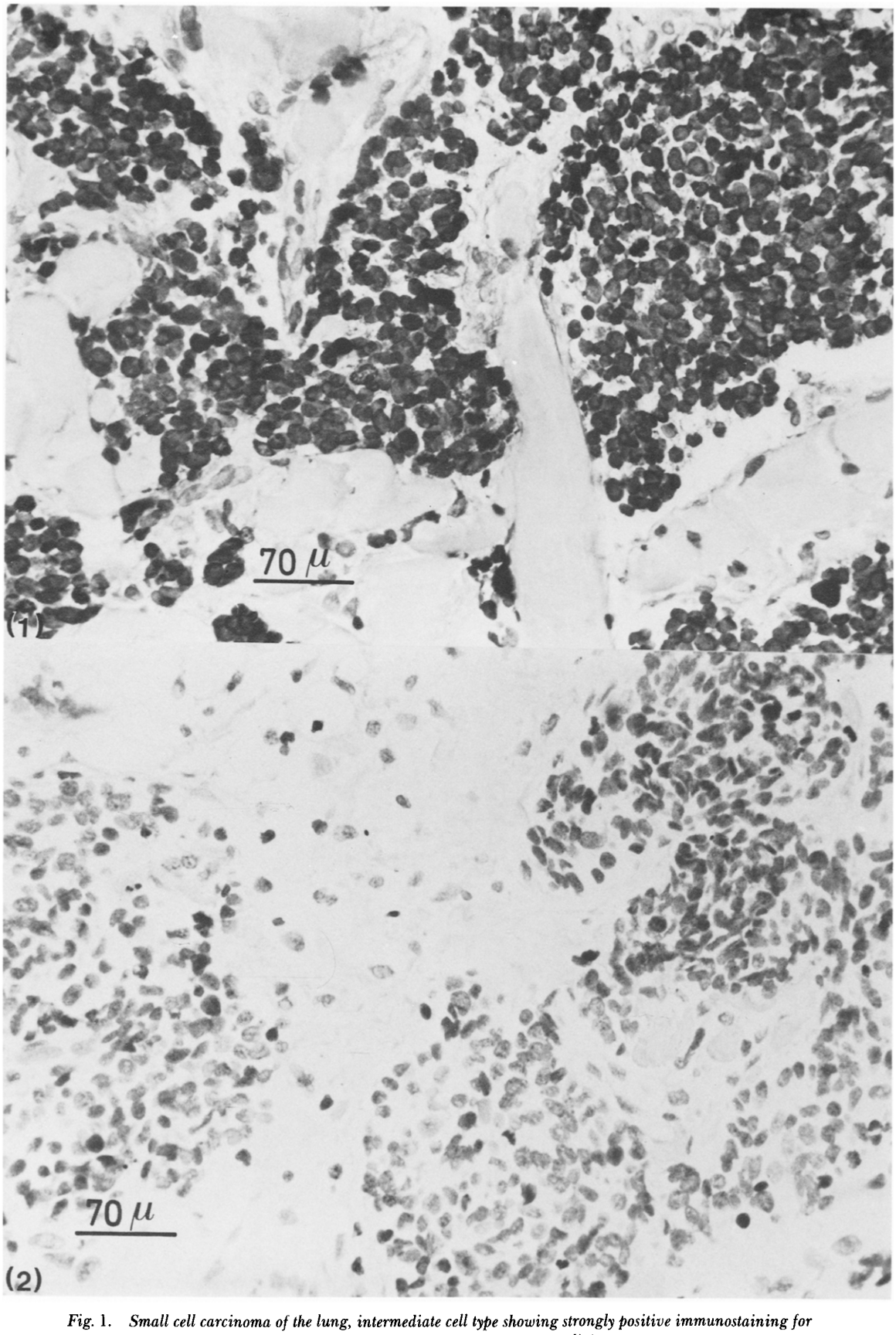


*Fig. 1. Small cell carcinoma of the lung, intermediate cell type showing strongly positive immunostaining for CEA. (CEA-PAP, counterstain haematoxylin).*

*Fig. 2. Same case as Fig. 1. Control section with antiserum to CEA absorbed with antigen. No immunostaining is observed. (Counterstain haematoxylin).*

control sections (Fig. 2). Non-specific background staining was minimal at the anti-CEA and PAP concentrations used. Staining of granulocytes and monocytes was absent after absorption of the commercial antiserum with the perchloric acid extraction of spleen. This indicated that the absorbed antiserum did not bind to the non-specific cross-reacting antigen of von Kleist [12].

Strongly positive staining for CEA was found in biopsies from 13 patients. Slightly positive staining was observed in 22 patients, while negative staining results were obtained in 18 patients. Strongly positive staining for CEA was found in 28% of bronchial biopsies and 21% of biopsies from metastases. Slightly positive staining was observed in 41% of bronchial biopsies and in 42% of biopsies from metastases. Negative staining was observed in 31% of bronchial biopsies and in 37% of metastases.

*Immunostaining results compared with survival*

The 3 groups of patients with strongly positive, slightly positive and negative staining results for CEA were comparable with respect to chemotherapeutic treatment protocols and to initial performance status. The initial staging results of the 3 groups together with the median survivals are shown in Table 1. The group of patients with a strongly positive staining reaction to CEA had a median survival of 338 days (range, 83–688 days), as compared with 226 days (range, 35–443 days) in the group with negative staining results. Statistical analysis showed that the different survival between these 2 groups was significant ($P < 0.05$). When only patients whose diagnosis was based on bronchial biopsy were considered, the difference in survival between the strongly positive and negative groups was also significant ($P < 0.01$). However, no significant difference in survival was found between patients with strongly positive and negative biopsies from metastases.

*Immunostaining results compared with WHO 1977 subclassification*

Histological subclassification according to WHO 1977 [8] was possible in 38 biopsies. Twenty-three biopsies were of the oat-cell subtype. Eight of these (35%) were strongly positive for CEA and 9 (39%) were negative. Twelve biopsies were of the intermediate subtype. Two of these were strongly positive (17%) and 5 (42%) were negative. All 3 biopsies of the combined subtype were slightly positive. No statistically significant difference was observed between the staining results for CEA in the oat-cell and intermediate subtypes.

## DISCUSSION

The present study demonstrated that 25.4% of patients with SCCL had a strongly positive staining reaction for presence of CEA in pretreatment diagnostic biopsies. The study was based only on diagnostic biopsies which were either from the primary tumour or from metastases. It was therefore not possible to examine whether patients with strongly positive bronchial biopsies also had strongly positive metastases and vice versa. However, the ratio of positive and negative staining results were similar in biopsies from the primary tumours and from metastases.

A significantly longer median survival was observed from patients with strongly positive staining results compared to patients with negative staining results. The fact that no significant difference in survival was found in the subgroups of patients with strongly positive and negative staining in biopsies from metastases might be explained by the difference in initial staging results (as shown in Table 1) in these 2 groups. The reason for the difference in survival between patients with strongly positive and negative biopsies is unknown. The difference cannot be attributed to initial staging results, initial performance status or chemotherapeutic regimens.

Other authors have conducted immunoperoxidase studies on the presence of CEA in the following types of carcinoma: epidermoid carcinoma of the lung, adenocarcinoma of the lung, carcinoma of the breast, carcinoma of the bladder and adenocarcinoma of the colon. It is interesting that these authors have stated that positive staining for CEA is more often found in higher than in lower differentiated tumours[5, 6, 13–15]. In contrast to most other malignant epithelial tumours, SCCL is not graded histomorphologically in highly and moderately differentiated and undifferentiated forms. A possible explanation for the longer median survival shown in the present study, for patients with biopsies strongly positive for CEA as compared with patients with negative biopsies, could be that strongly positive immunoperoxidase staining for CEA in SCCL denotes a higher grade of tumour differentiation than negative staining. In this context it is interesting that no correlation was found

*Table 1. Pretreatment biopsy*

| | Strongly positive | | | Slightly positive | | | Negative | | |
|---|---|---|---|---|---|---|---|---|---|
| | Bronchial biopsy | Metastasis | Total | Bronchial biopsy | Metastasis | Total | Bronchial biopsy | Metastasis | Total |
| Total no. of patients | 8 | 5 | 13 | 12 | 10 | 22 | 9 | 9 | 18 |
| Patients with initial regional disease | 5 | 1 | 6 | 5 | 4 | 9 | 5 | 4 | 9 |
| Patients with initial extensive disease | 3 | 4 | 7 | 7 | 6 | 13 | 4 | 5 | 9 |
| Median survival (days) | 358* | 282 | 338† | 293 | 239 | 286 | 189* | 277 | 226† |
| Range | 201–688 | 83–444 | 83–688 | 68–763 | 45–478 | 45–763 | 35–443 | 138–399 | 35–443 |

*$P < 0.01$.

†$P < 0.05$.

Initial staging and survival of patients with small cell carcinoma of the lung grouped according to tissue staining results for presence of CEA.

between immunoperoxidase staining for CEA and morphological subtypes according to the WHO 1977 classification.

Whether presence of CEA in tumour cells in SCCL can be used as a prognostic factor in patients treated with intensive chemotherapy will require further study on carefully matched patients using multifactorial analysis.

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