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Esophageal carcinoma-associated proteins detected by two-dimensional polyacrylamide gel electrophoresis

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SUMMARY

Patterns of proteins of five surgically resected esophageal carcinomas were studied by two-dimensional polyacrylamide gel electrophoresis with silver staining. The samples of normal esophageal mucosa and esophageal carcinoma from the same patient were compared. Each gel had ca. 300 protein spots and had a similar pattern of proteins. Four spots were observed in all of the esophageal carcinomas that were not present in any of the normal mucosae. The molecular weights and isoelectric points were 46 000 and 5.3, 46 000 and 5.2, 36 000 and 4.7 and 33 000 and 5.1, respectively. One spot was observed in all of the normal mucosae but not in any of the esophageal carcinomas. Its molecular weight and isoelectric point were 27 000 and 5.3, respectively.

INTRODUCTION

Over 90% of primary malignant tumours of the esophagus are squamous cell carcinomas. Esophageal carcinoma is a silent tumour in its early stage and usually diagnosed at the advanced stage. Recently squamous cell carcinoma-related antigen TA-4 has been investigated as a tumour marker of esophageal carcinoma [1]. It was extracted from squamous cell carcinoma of the uterine cervix [2].

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of pro-

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teins has been developed since 1975, mainly owing to O'Farrell [3], and has recently become a popular technique for detecting potential markers. Several groups have compared normal and transformed cells in culture [4–7], but little work has been done with solid tumours because of the difficulty in obtaining homogeneous cell populations.

The present study was carried out to identify the additional minor proteins in esophageal cancer tissue, using 2D-PAGE combined with silver staining.

EXPERIMENTAL

Chemicals

Phenylmethylsulphonyl fluoride, pepstatin, benzamidine and Nonidet P-40 were purchased from Sigma (St. Louis, MO, U.S.A.). Acrylamide, N,N′-methylenebisacrylamide, ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine, trisaminomethane, glycine, urea, sodium dodecylsulphate (SDS) and silver reagent were purchased from Wako (Osaka, Japan). Coomassie brilliant blue R-250 (CBB) and 2-mercaptoethanol were purchased from Nakarai Chemicals (Kyoto, Japan). Ampholine (pH 3.5–9.5) was purchased from LKB (Uppsala, Sweden).

Specimen acquisition

Five patients with esophageal carcinoma, all men between 58 and 71 years (mean 65.6 years) were examined. All five esophageal carcinomas were diagnosed as squamous cell carcinoma histologically by endoscopic biopsy. Specimens of esophagus, obtained at surgery from patients with esophageal carcinoma, were processed without delay. Small pieces (ca. $0.5 \times 0.5 \times 0.3$ cm) of tumour and normal mucosa were sectioned by blunt and sharp dissection. Half of each specimen of tumour and normal mucosa was stored at $-70°C$, generally within 10 min of their arrival in the pathology laboratory of our hospital. The other half of each specimen was pinned to a cork plate to approximate its original size and floated in 10% buffered formalin. Thin sections of the formalin-fixed mucosa were cut and stained with hematoxylin and eosin for histological examination.

Sample preparation for electrophoresis

Samples were prepared for electrophoresis by a modification of the method of Tracy et al. [8]. To date, five paired samples have been analysed. Initial preparation was done in a Petri dish. Frozen tissue was shaved with a cold scalpel, until 100–200 mg had been shaved off. This was rapidly weighed and placed in a cold test-tube, and homogenization buffer (8 mol of urea and 50 ml of 2-mercaptoethanol per litre) was added, 600 μl per 150 mg of frozen tissue. In order to inhibit proteolysis, 10 μl of each of two solutions (25 mg of phenylmethylsulphonyl fluoride plus 1 mg of pepstatin per 1.4 ml of ethanol and

16 mg of benzamidine per 1 ml of water) were added to 150 mg of frozen tissue. This was immediately homogenized (Econo-grind homogenizer; Radnoti Glass Technology, Monrovia, CA, U.S.A.). After centrifugation in this homogenizer (1640 *g*, 10 min) 100 μl of the supernatant fluid were removed for further processing, and the remainder was frozen. The sample was placed in a micro-size ground-glass homogenizer, and 33 μl of neutralization buffer [containing, per litre, 8 mol of urea, 80 ml of Nonidet P-40 surfactant and 50 g of ampholine (pH 3.5–9.5)] were added. The sample was again homogenized for 20 s, and then centrifuged at 15 860 *g* for 1 h (high-speed microcentrifuge MC-150; Tomy Seiko, Tokyo, Japan). A 100-μl Syringe (Hamilton, Reno, NV, U.S.A.) was then used to aspirate 50 μl from the middle of the tube, and 40 μl of the sample were immediately applied to the isoelectric focusing gel and electrophoresed. In the case of a patient, 25 μl each of the normal and tumour samples were mixed together and then electrophoresed.

Electrophoresis

The 2D-PAGE system of O'Farrell [3] was used, with some modifications. This system depends on isoelectric focusing under dissociating conditions (per litre, 8.5 mol of urea, 20 g of Nonidet P-40 surfactant and 20 g of pH 3.5–9.5 ampholine) in the first dimension and SDS gel electrophoresis in the second dimension. In isoelectric focusing, the gels were prerun at 200 V for 2 h, and then run at 300 V for 15 h and at 500 V for 1 h. In SDS gel electrophoresis, the stock gels were run at 24 mA and the analytical gels were run at 30 mA. The finished gels were stained with the silver reagent, as previously described by Inoue et al. [9], and photographed. All of the gels were pictured with the alkaline side on the left. One of the electrophoresed gels was stained with CBB and later doubly stained with the silver reagent.

RESULTS

Histological diagnosis

All of the normal esophageal mucosae and carcinomas were examined histologically. The normal esophageal mucosa consisted of epithelium, lamina propria mucosa, lamina muscularis mucosa and submucosa (Fig. 1A). The lamina propria consisted of the loose connective tissue with relatively thin collagenous fibres. The muscularis mucosae consisted of longitudinal smooth muscle fibres and thin elastic networks. The dense connective tissue of the submucous layer consisted of collagenous and elastic fibres and small infiltrations of lymphocytes around the glands. In all of five tumours, one tumour was well differentiated squamous cell carcinoma and four were moderately differentiated squamous cell carcinomas (Fig. 1B). These tumours exhibited various degrees of cancerous invasions from submucosa to adventitia.

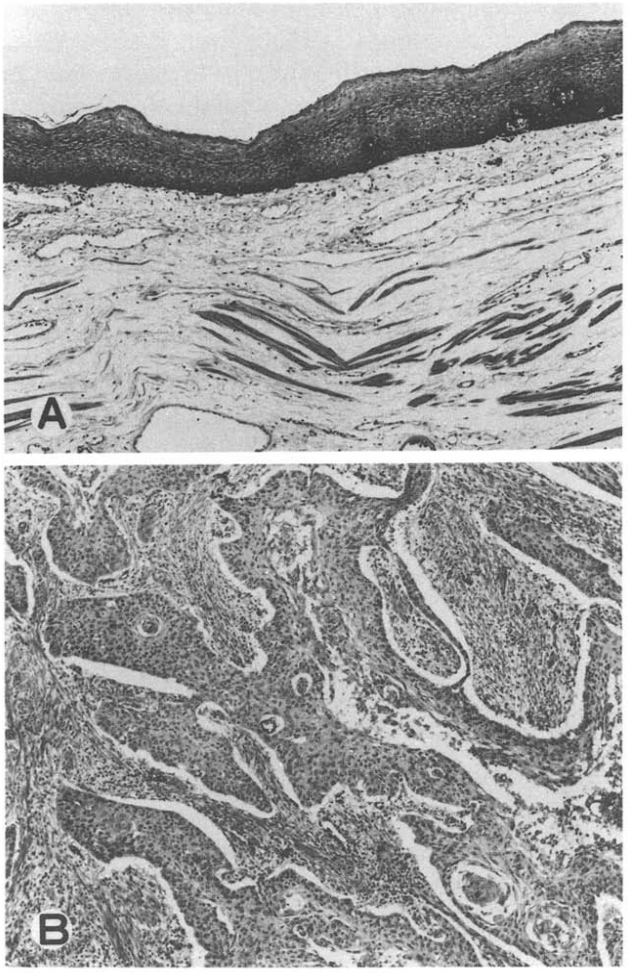


Fig. 1. Histology of esophageal mucosa and carcinoma. Histological sections prepared and stained as described in the text: (A) normal esophageal mucosa; (B) esophageal squamous cell carcinoma.

Electrophoretic comparison of proteins of normal mucosa and squamous cell carcinoma

Samples of normal mucosa and squamous cell carcinoma from the same patient were electrophoresed. The results of one experiment is shown in Fig. 2. A fixed amount of protein in samples was ca. 1 mg, as determined by the method of Lowry et al. [10]. About 300 spots appeared in the each gel, and the majority of spots on both gels were identical. Fig. 3 illustrates a comparison of the same gel at higher magnification. There were several differences. One spot was present in the outlined area a of the normal sample but absent in the tumour sample (arrows, panel A). Four spots were present in the tumour sample but absent

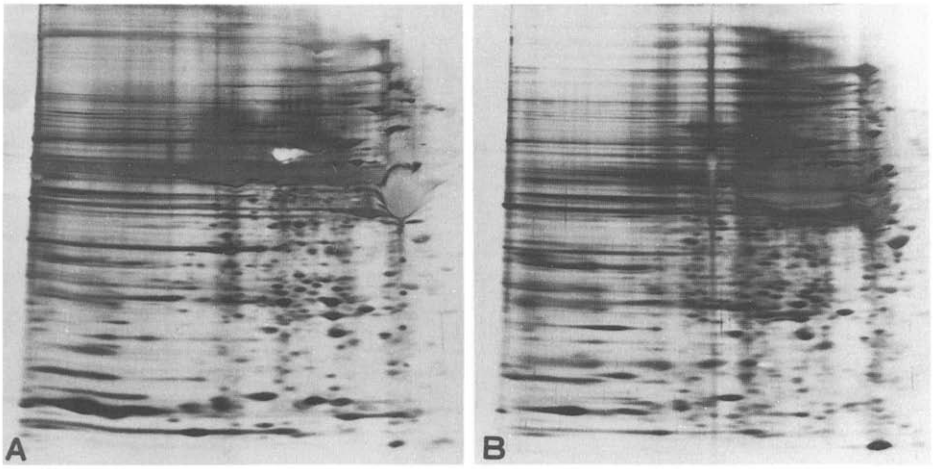


Fig. 2. Electrophoretic patterns of normal mucosa and carcinoma. Two-dimensional gels of (A) normal mucosa and (B) squamous cell carcinoma from the same patient.

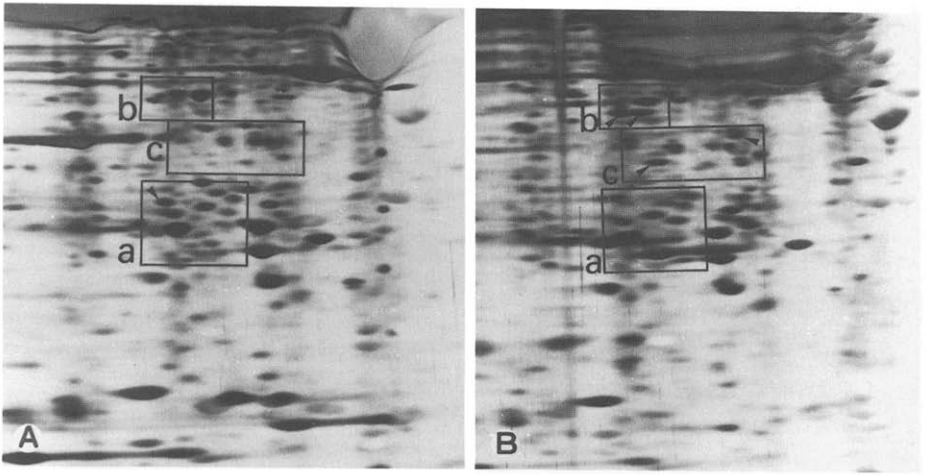
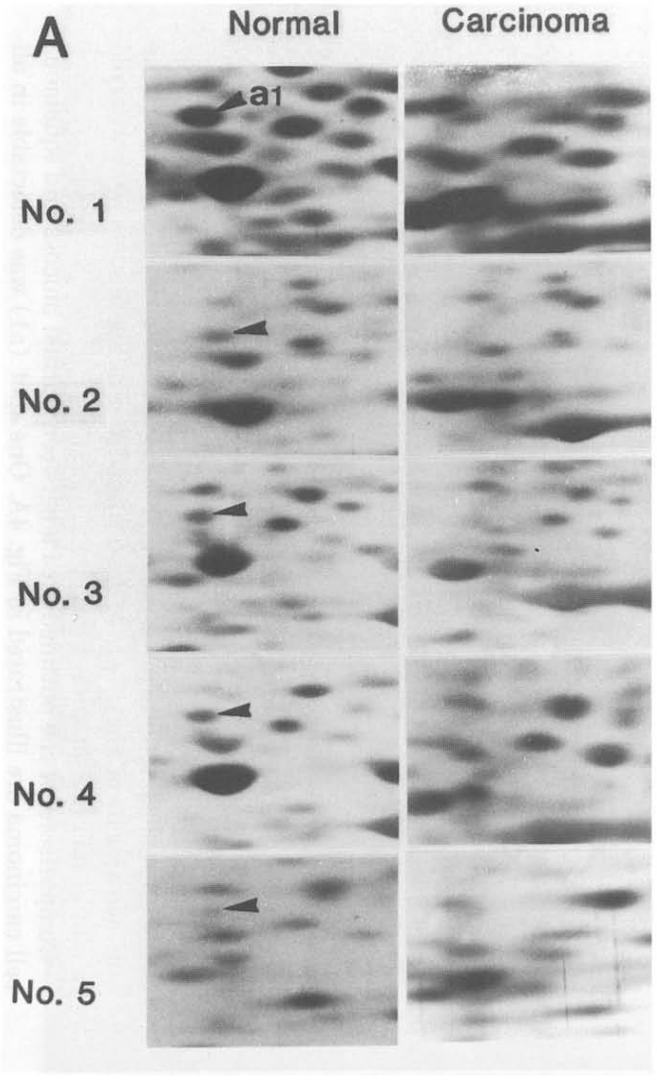


Fig. 3. The patterns of Fig. 2, at higher magnification. One spot in the outlined ares a of the normal samples is absent from the tumour samples. Four spots in the outlined areas b and c of the tumour samples are absent from the normal samples.

in the normal sample (arrows, panel B). Two of these spots were found in the outlined area b and the others in the outlined area c. These spots were observed in all five pairs of samples.

Comparisons of the outlined area a between normal mucosa and squamous cell carcinoma are illustrated in Fig. 4A. One spot (a1) was detectable in all


A
Normal
Carcinoma
a1
No. 1
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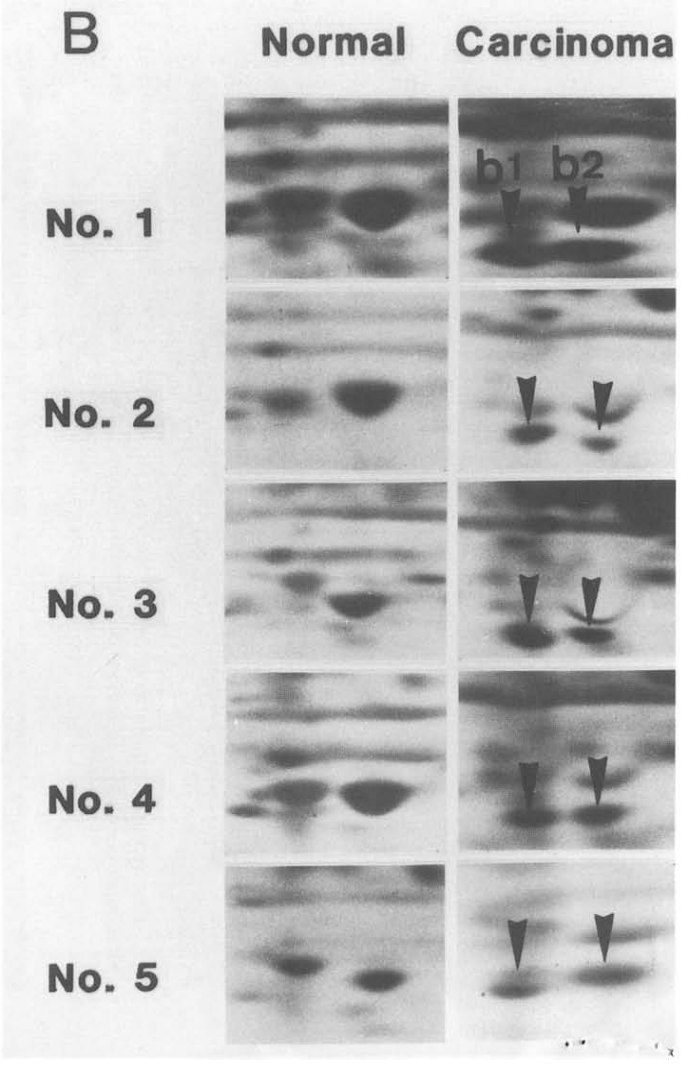
B
Normal
Carcinoma
b1
b2
No. 1
No. 2
No. 3
No. 4
No. 5

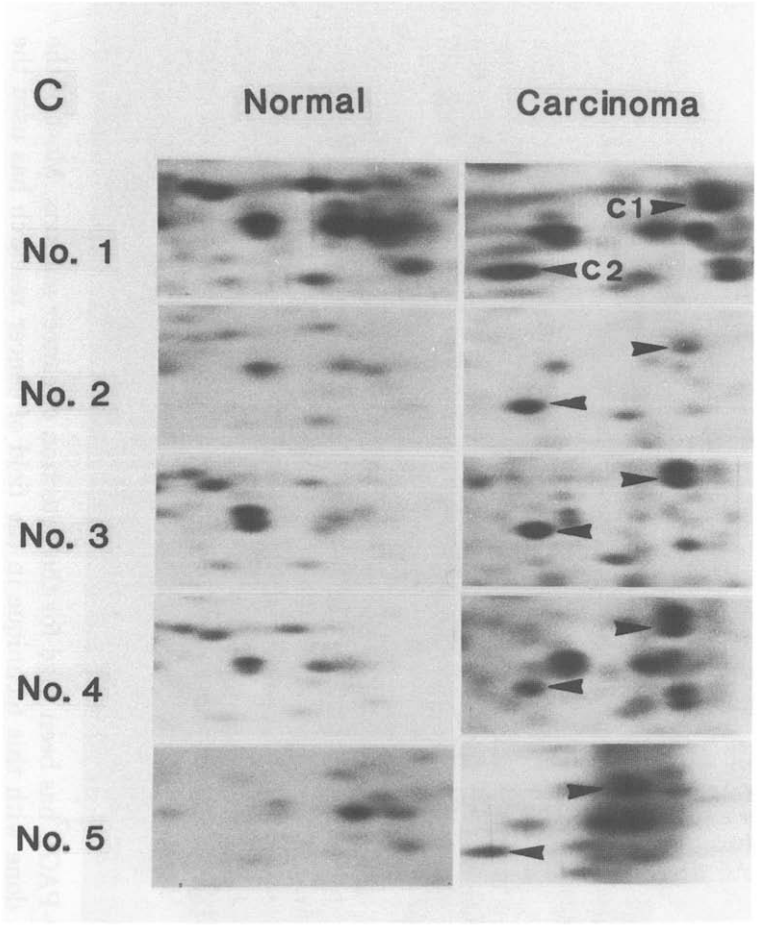

Fig. 4. Electrophoretic comparison between normal mucosa and carcinoma, from samples taken from all five patients. A, B and C correspond to the outlined areas a, b and c in Fig. 3, respectively. One spot (a1) is detectable in all the normal samples but not in any of the tumour samples (A). Four spots (b1, b2, c1 and c2) are detectable in all the tumour samples but not in any of the normal samples (B and C).

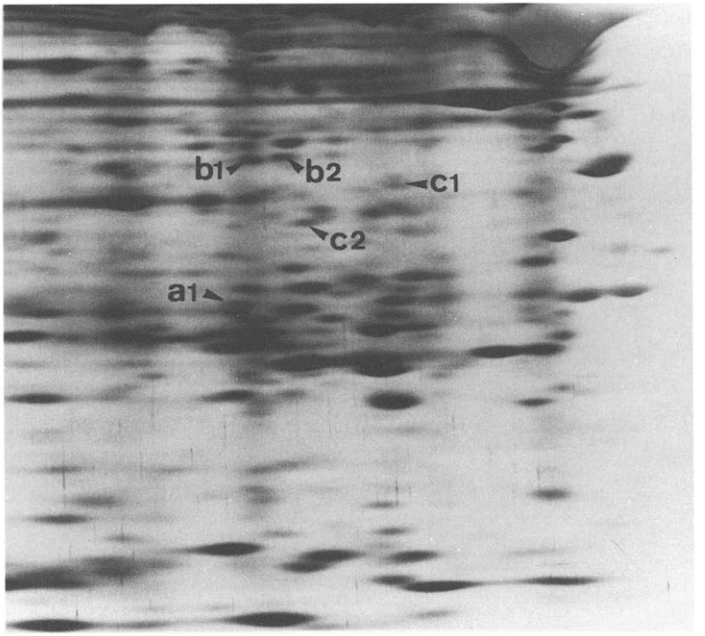

Fig. 5. Electrophoretic pattern of a mixture of normal and tumour samples, taken from the same patient. All five spots are present.

the normal samples but not in any of the tumour samples. The outlined areas b and c are shown in Fig. 4B and C, respectively. Four spots (b1, b2, c1, and c2) were detectable in all the tumour samples but not in any of the normal samples.

The gel for the mixture of the normal and tumour samples from the same patient is shown in Fig. 5. In this gel, all five spots were observed. The molecular weights and isoelectric points of these spots were, respectively: a1, 27 000 and 5.3; b1, 46 000 and 5.3; b2, 46 000 and 5.2; c1, 36 000 and 4.7; c2, 33 000 and 5.1.

The gel of the tumour sample stained with CBB and double-stained with CBB and silver are illustrated in Fig. 6. In the gel stained with CBB two spots (c1 and c2) were visible, but the other two spots (b1 and b2) were invisible (arrows, Fig. 6). The gel of the tumour sample shown in Fig. 7 was stained with CBB and arranged with the isoelectric focusing gel on the upper side and the SDS polyacrylamide gel on the left side. About 70 spots were found in the gel stained with CBB.

DISCUSSION

2D-PAGE has been used for the detection of cancer markers. Most of the work done with this technique in the field of cancer research has used the

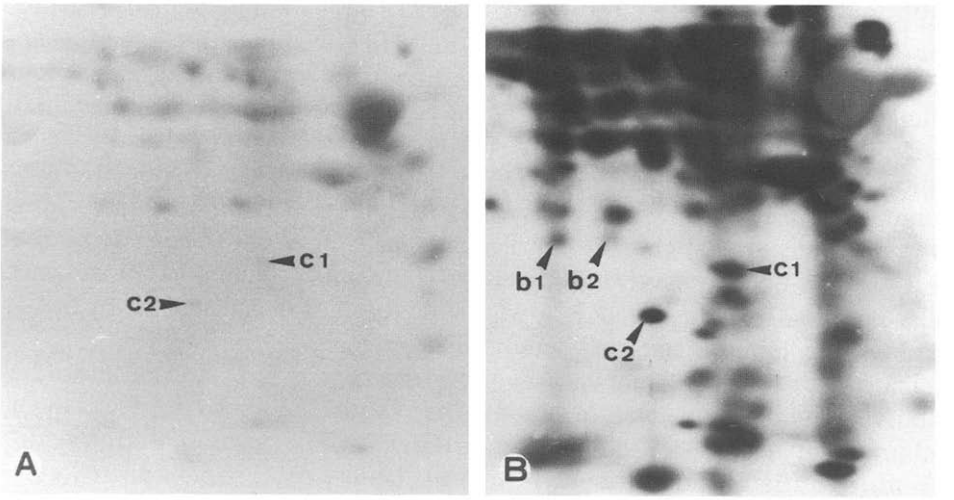

Fig. 6. Electrophoretic patterns of samples stained with (A) CBB and with (B) CBB and silver. In the gel stained with CBB two spots (c1 and c2) are visible, but the other two spots are invisible (A).

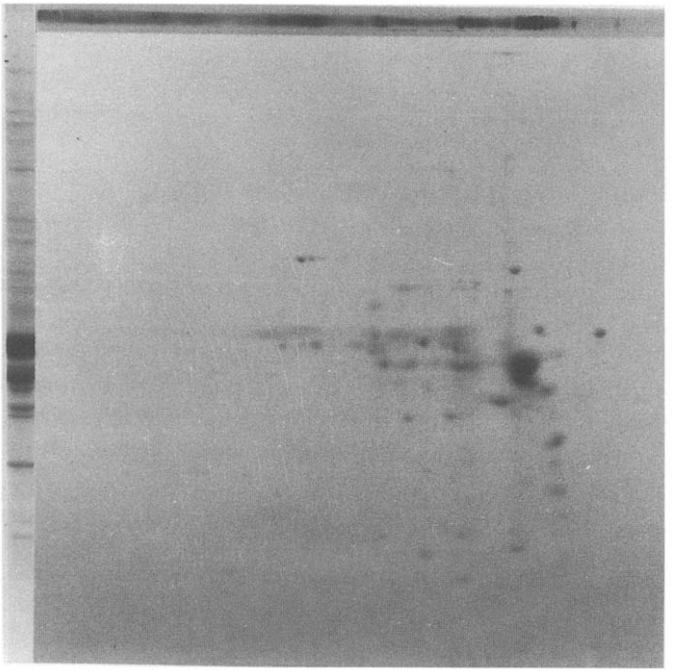

Fig. 7. Electrophoretic pattern of a tumour sample stained with CBB. The gel is arranged with the isoelectric focusing gel on the upper side and the SDS polyacrylamide gel on the left side.

ability of this system to separate and compare a large number of proteins. This has led to the identification of a large number of transformation-specific peptides. Wu et al. [11] compared the nuclear proteins of several human tumour cells and several normal cells by 2D-PAGE. They formed two peptides in all the tumour cells but in none of the normal cells. Scheele [12] analysed proteins contained in pancreatic juice by 2D-PAGE. Analysis of the pancreatic juice obtained from a patient with pancreatic cancer showed a number of additional proteins that were not observed in the pancreatic juices obtained from normal persons. In this way, several groups have compared normal and transformed cells, but little work has been done with solid tumours because of the difficulty in obtaining homogeneous cell populations.

Thorsrud et al. [13] mapped the pattern of proteins in normal mucosae, tubular adenomas and colon carcinomas by 2D-PAGE. They reported that tubular adenomas and carcinomas had strikingly similar protein patterns, different from that of normal mucosae. In the same way, Tracy et al. [8] compared patterns of normal colon mucosae and colon adenocarcinomas by 2D-PAGE. They observed five spots in the tumour sample that were not detectable in the normal sample.

Little work on esophageal carcinoma has been done directly by 2D-PAGE. In this work, we have attempted to compare samples of esophageal normal mucosa and squamous cell carcinoma from the same patient with esophageal carcinoma. It was demonstrated in all five cases studied that four spots were present in the gels of tumour samples but not in the gels of normal samples. These spots appear to be tumour-associated proteins. The exact relationship of most of these tumour-associated proteins to the transformation process, however, remains vague.

On the other hand, all samples of the normal esophageal mucosae showed a protein that was not detected in the esophageal carcinoma samples. The mechanism of this protein is unknown. It has been demonstrated that one type of protein disappeared in the transformed cell membranes [14–16]. Even if the normal mucosa-associated protein could be resolved by proteinase, it would bear no relation to the tumour-associated proteins, because the molecular weights of the tumour-associated proteins are higher than that of a normal mucosa-associated protein. In our study, a proteolytic inhibitor was added to the sample preparation. If the proteolytic inhibitor had acted inadequately, many more changes would have been found.

The proteins that we have observed undergo qualitative pattern changes during staining. The next stage of our investigations will be to extract these proteins from gels and determine the amino acid sequences.

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REFERENCES

1 M. Kitamura, H. Matsuda, H. Matsuura, H. Kai, H. Ageo and K. Sugimachi, Jpn. J. Surg., 86 (1985) 1561.
2 H. Kato and T. Torigoe, Cancer, 40 (1977) 1621.
3 P.H. O'Farrell, J. Biol. Chem., 250 (1975) 4007.
4 G. Milman, E. Lee, G.S. Ghangas, J.R. McLaughlin and M. George, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 4589.
5 R.A. Steinberg, P.H. O'Farrell, U. Friedrich and P. Coffino, Cell, 10 (1977) 381.
6 M. Strand and J.T. August, Proc. Natl. Acad. Sci. U.S.A., 74 (1977) 2729.
7 H.G. Wada, J.E. Shindelman, A.E. Ortmeyer and H.H. Sussman, Int. J. Cancer, 23 (1979) 781.
8 R.P. Tracy, L.E. Wold, R.M. Currie and D.S. Young, Clin. Chem., 28 (1982) 915.
9 Y. Inoue, H. Asaga and M. Tamura, Physico-Chem. Biol., 30 (1986) 229.
10 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 1951.
11 B.C. Wu, W.H. Spohn and H. Busch, Cancer Res., 41 (1981) 336.
12 G.A. Scheele, Cancer, 47 (1981) 1513.
13 A.K. Thorsrud, M.H. Vatn and E. Jellum, Clin. Chem., 28 (19892) 884.
14 A. Vaheri and E. Ruosiahti, Int. J. Cancer, 13 (1974) 579.
15 R.O. Hynes, Biochim. Biophys. Acta, 458 (1976) 73.
16 K. Olden and K.M. Yamad, Cell, 11 (1977) 957.