ELSEVIER

Journal of Chromatography B, 667 (1995) 153–160

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Comparison of membrane proteins from benign and malignant human thyroid tissues by two-dimensional polyacrylamide gel electrophoresis

J.D. Lin[a,*], C.C. Huang[a], H.F. Weng[a], S.C. Chen[b], L.B. Jeng[b]

[a]*Division of Endocrinology and Metabolism, Department of Medicine, Chang Gung Memorial Hospital, 5 Fu-Shin St. Kweishan county, Taoyuan Hsien, Taipei, Taiwan*

[b]*Department of Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan*

First received 8 September 1994; revised manuscript received 19 December 1994; accepted 29 December 1994

Abstract

In this study two-dimensional (2D) polyacrylamide gel electrophoresis with silver staining was used to analyze cellular membranous proteins of various normal and pathological human thyroid tissues. The aim was to understand the differences in cellular membranous proteins between these tissues, which would aid in the differential diagnosis of thyroid malignancy. Characteristic protein spots had a molecular mass of 50–64 kDa and a p*I* of 5.7–6.5. There were two groups of isoform protein spots in this area. The higher-molecular-mass group was found in follicular thyroid cancer tissues which and was not visible in normal thyroid tissues. The low-molecular-mass group was found in follicular carcinoma or adenoma tissues and was detected in one to three spots. The papillary thyroid carcinoma tissues gave different 2D gel maps. There were few spots of papillary thyroid carcinoma tissue membranous proteins within the examined area. The 2D gel maps may be used for differential diagnosis of follicular neoplasm. The characteristics of these protein spots require further investigation.

1. Introduction

Thyroid neoplasm is the most common neoplastic disorder encountered in endocrine clinics. The neoplasm usually occurs with painless thyroid non-toxic nodules [1,2]. Although approximately 4% of the general population is affected by thyroid nodules [3], the mortality of the disorder is not high. Most of these thyroid nodules are benign. In the histopathology of thyroid malignant neoplasms, there are three different types with thyroid follicular cell origin: papillary carcinoma, follicular carcinoma and anaplastic carcinoma. Medullary carcinoma is the distinct type of thyroid carcinoma with a parafollicular cell origin. In clinical practice, we use non-invasive thyroid scans, thyroid echo, and invasive aspiration cytology or biopsy procedures to differentiate malignant nodules from benign nodules [4–7]. For follicular thyroid carcinoma there is no special examination tool which can differentiate malignant from benign nodules. Even with thyroid biopsy, follicular carcinoma is sometimes difficult to differentiate from follicular adenoma. Until now, we had no specific

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00002-X

tumor marker for pre-operative diagnosis of thyroid follicular carcinoma.

Two-dimensional (2D) gel electrophoresis is a powerful tool which gives a finger print of each cellular protein [8,9]. From the analysis of 2D gels of benign and malignant thyroid follicular cellular proteins, we can find the specific polypeptides in malignant cells [10,11]. From the 2D gel, we can determine the molecular mass and isoelectric point (p*I*) of the specific polypeptides. In this study, we applied 2D gel electrophoresis to the cellular membranous proteins of normal, benign, and malignant thyroid tissues. Our objective was to identify proteins as tumor markers for clinical diagnosis and to determine prognosis.

2. Experimental

2.1. Chemicals and reagents

Sodium dodecyl sulphate (SDS), acrylamide N,N′-methylene-bisacrylamide (Bis), glycine, bromophenol blue, silver stain kit, β-mercaptoethanol, urea, ampholine (pH 3–10 and pH 5–8), low-molecular-mass standard were purchased from Bio-Rad (Richmond, CA, USA). Phenylmethylsulphonyl fluoride, pepstatin, benzamidine, phosphoric acid and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA).

2.2. Specimen acquisition

Twenty samples of normal and benign thyroid tissues, including nodular hyperplasia and follicular adenoma tissues, were used as specimens. Ten samples of malignant thyroid tissues, including papillary and follicular carcinoma, were collected during individual operation (Table 1). All tissues were verified by histopathology. After the specimens were washed three times with PBS to remove the blood, they were stored at −75°C until analysis.

2.3. Preparation of samples for electrophoresis

Samples were prepared for electrophoresis by a modification of the method of Bjorkman et al. [12]. About 200 mg of frozen tissue was scraped with a cold scalpel. Before the tissues were homogenized over ice (Econo-grind homogenizer; Radnoti Glass Technology, Monrovia, CA, USA), proteinase inhibitors (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 the 200 mg frozen tissue. After homogenization, the samples were centrifuged (1640 *g*, 10 min) and the supernatant was removed for further centrifugation. The samples were again centrifuged (40 000 *g*, 1 h). The pellet with membrane proteins was then dissolved in PBS containing proteinase inhibitors as described above. The protein concentration was measured by the Bio-Rad (Richmond, CA, USA) protein micro-assay procedure using BSA as a standard.

Table 1
Human thyroid tissues used in two-dimensional gel electrophoresis

Tissue	Number
Normal	7
Nodular hyperplasia	8
Follicular adenoma	5
Follicular carcinoma	4
Papillary carcinoma	6

2.4. Electrophoresis

The 2D-PAGE system of O'Farrell was used with some modification [13]. A 80-μg amount of each sample was saturated with solid urea and 1 μl of solution containing 0.1 ml of 10% SDS, 0.02 ml of Bio-Lyte pH 3–10 ampholyte, 0.18 ml of pH 5–7 ampholyte, 0.1 ml of β-mercaptoethanol, and 0.2 ml of Triton X-100 were added to 5 μl of the sample. For the first dimension isoelectric focusing (IEF) electrophoresis gels were cast in 180 × 1.0 mm I.D. glass tubes. For IEF, the gels were prerun at 200 V for 2 h, at 400 V for 18 h, and at 800 V for 2 h. After running the IEF gels, they were equilibrated in SDS reducing buffer for 1 h, and embedded in second dimension gels. The pH gradients were measured by laying two rod gels on a piece of glass and cutting them into twelve

1-cm segments. Each pair of segments was eluted in 0.5 ml of distilled, de-ionized, degassed water and shaken for 15 min. The pH of the eluate was subsequently measured with a standard pH meter. For the second dimension, 12.5% discontinuous polyacrylamide slab gel electrophoresis (PAGE) was used. PAGE was run under a constant voltage of 200 V until the dye front reached the bottom of the gels. Ten μl of low range SDS-PAGE standards (Bio-Rad; Richmond, CA, USA) were loaded on the right margin of the second dimension gels. After running, the gels were stained with the silver stain method. The staining kit was purchased from Bio-Rad.

3. Results and discussion

Seven thyroid tissues were removed from spots without cancer cell invasion of thyroid cancer patients for comparison. For the same amount of membranous protein from thyroid tissue, less protein spots were found in the normal thyroid tissue 2D maps as compared with pathological tissues (Fig. 1). The 2D maps revealed more spots for the nodular hyperplasia (Fig. 2) and adenomatous tissues (Fig. 3). In 2D gel electrophoresis of follicular thyroid carcinoma tissues, the protein spots with a molecular mass of 64 kDa to 50 kDa and a p*I* between 5.7 to 6.5 demonstrated characteristic patterns (Fig. 4). There were two groups of isoform protein spots with the same molecular mass but with different p*I* values. The higher-molecular-mass group–64 kDa (a1–a4 in Fig. 4)–presented in the 2D gel of follicular thyroid cancer tissues varied from 3 to 5 spots. In particular the a1 spot with a high p*I* was quite prominent. The lower-molecular-mass group–51 kDa (b1–b3 in Fig. 3)–varied from 1 to 3 spots in follicular thyroid adenoma tissues. These spots were not visible in normal thyroid 2D maps (Fig. 1). The protein spots from papillary thyroid cancer tissues (Fig. 5) had a characteristic pattern which was quite different from that of the follicular cancer tissues. In the same area, only a1 and b1 could be found in most papillary thyroid cancer tissues (Fig. 6).

The 2D maps from follicular adenoma (Fig. 3) revealed a pattern similar to that of follicular carcinoma (Fig. 4). In the five adenomatous tissue 2D maps (Fig. 7), two maps were similar to the carcinoma maps (Fig. 8). Very few 64 kDa molecular mass protein spots were found in the other three adenomatous tissues. In contrast to the 64 kDa spots, three low-molecular-mass 51

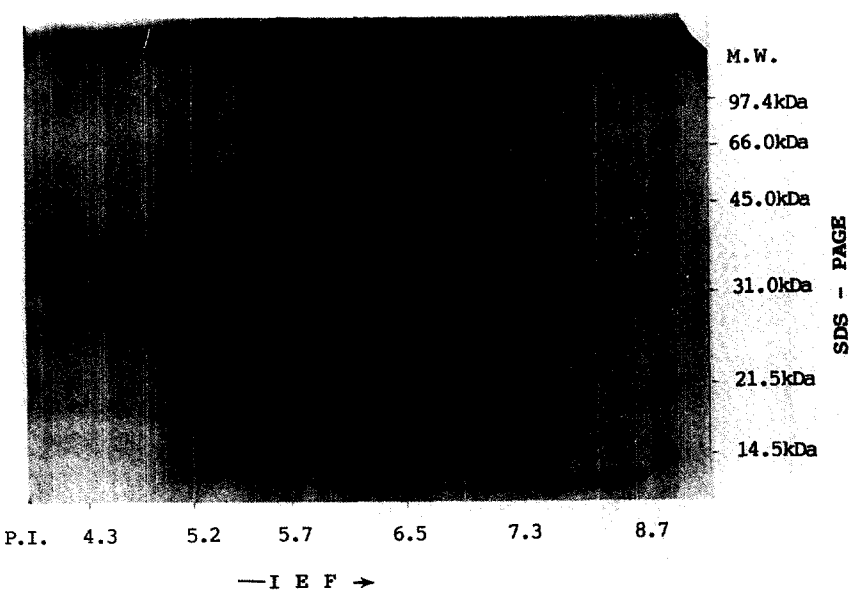

Fig. 1. Two-dimensional gel electrophoretic pattern of normal thyroid tissue.

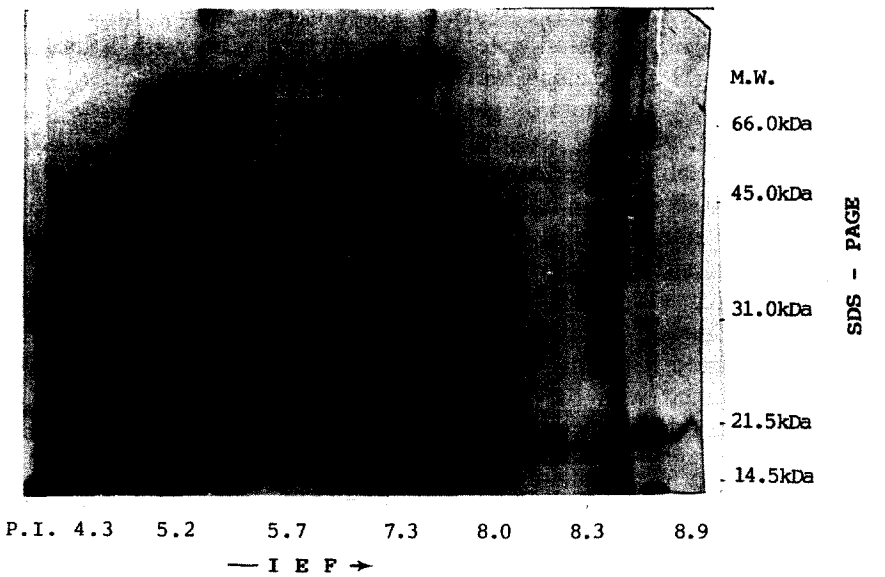

Fig. 2. Two-dimensional gel electrophoresis pattern of thyroid tissue with pathological proof of nodular hyperplasia.

kDa spots were observed more frequently in the adenomatous tissues (4 out of 5 tissues). The 2D maps from nodular hyperplasia revealed less spots (Fig. 9) when compared with follicular adenoma or carcinoma. The total 2D pattern of the nodular hyperplasia close resembled the maps from normal thyroid tissues.

In this study, 2D gel electrophoresis was used

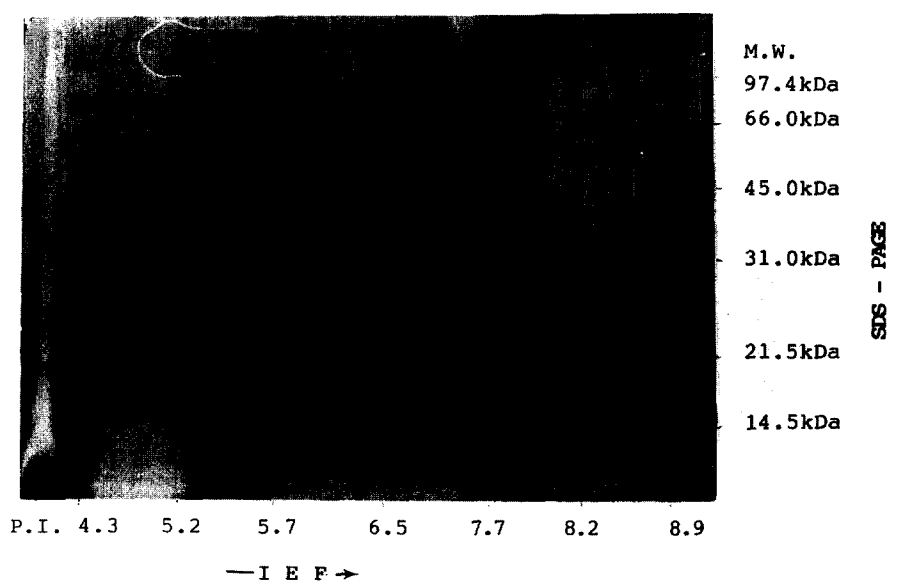

Fig. 3. Two-dimensional gel electrophoresis pattern of follicular thyroid adenoma tissue.

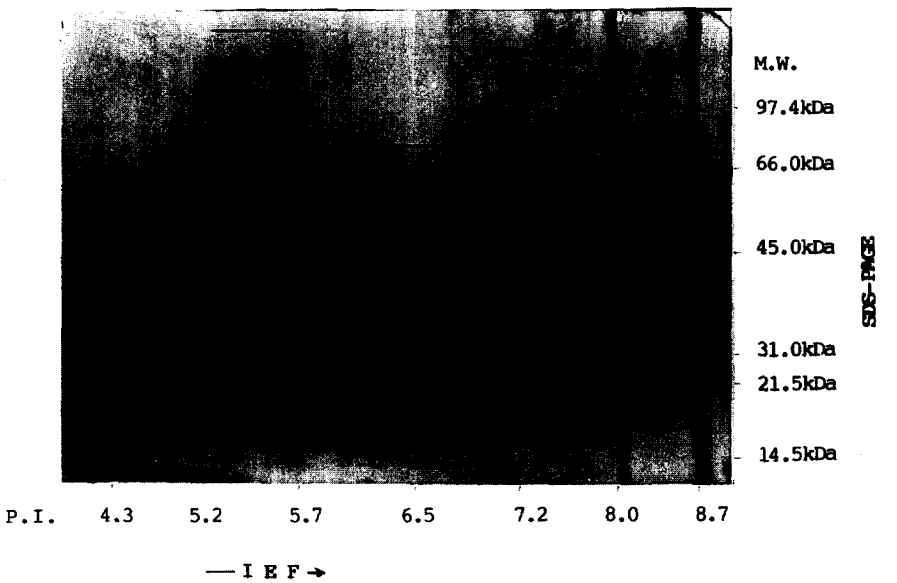

Fig. 4. Two-dimensional gel electrophoretic pattern of follicular thyroid carcinoma tissue. The arrows a1–a4 indicate isoform proteins which were specific for follicular thyroid cancer tissues.

to analyze the membranous proteins of various human thyroid tissues. Although the serum thyroglobulin level has been used as a post-operative, well-differentiated thyroid cancer tumor marker [14,15], the assay cannot be used for pre-operative diagnosis of thyroid carcinoma.

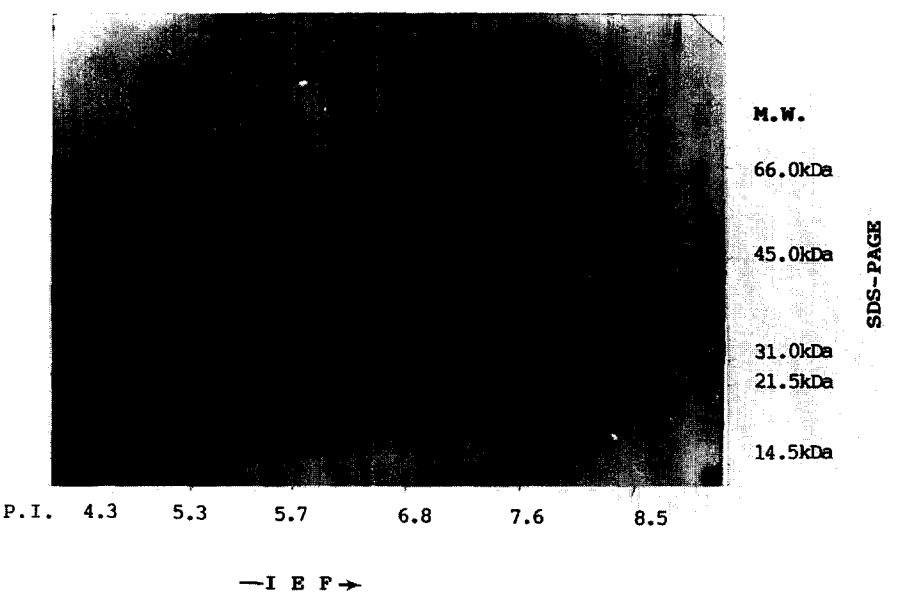

Fig. 5. Two-dimensional gel electrophoresis pattern of papillary thyroid cancer tissue.

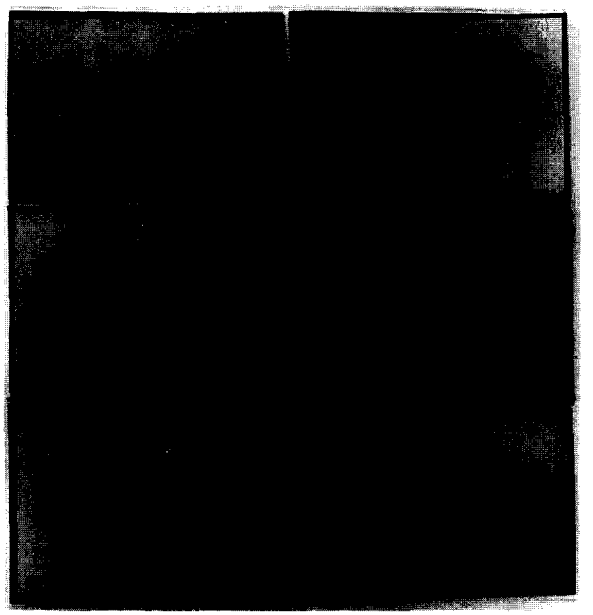

Fig. 6. Focal area of two-dimensional gel electrophoresis (molecular mass between 64 kDa to 50 kDa with a p*I* between 5.7 to 6.5) pattern of six papillary thyroid cancer tissues.

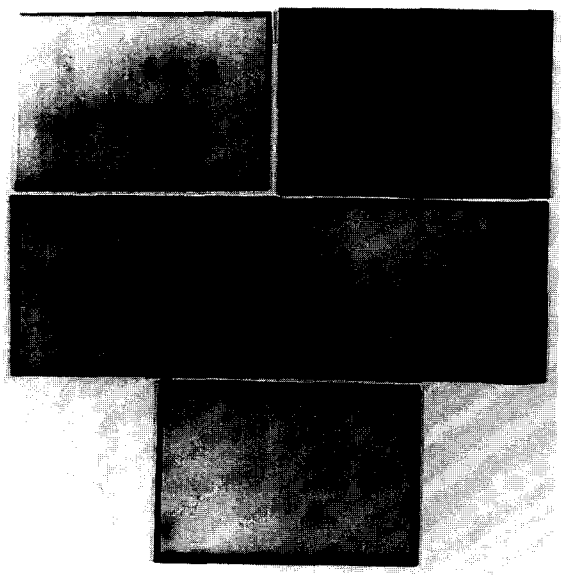

Fig. 7. Focal area of two-dimensional gel electrophoresis (molecular mass between 64 kDa to 50 kDa with a p*I* between 5.7 to 6.5) pattern of five thyroid follicular adenomas.

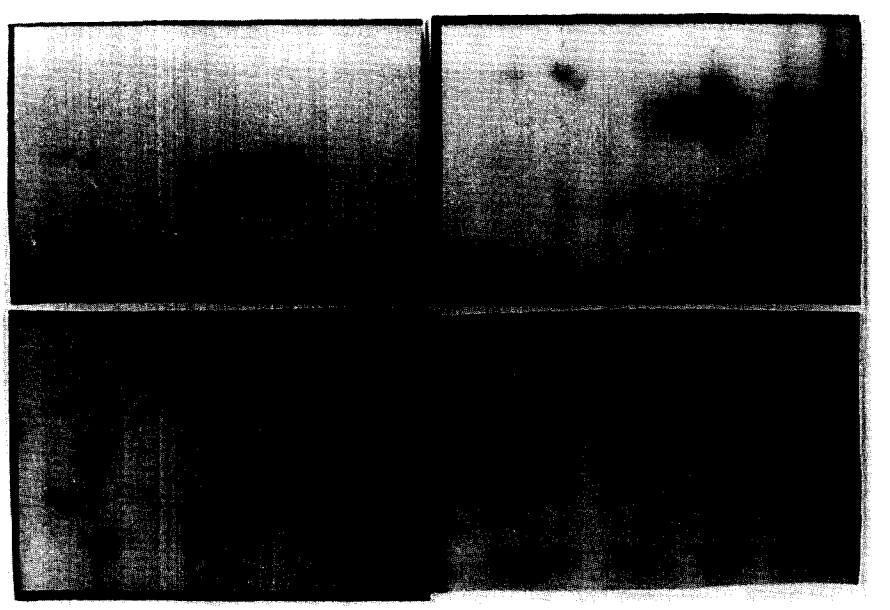

Fig. 8. Focal area of two-dimensional gel electrophoresis (molecular mass between 64 kDa to 50 kDa with a p*I* between 5.7 to 6.5) pattern of four follicular carcinomas.

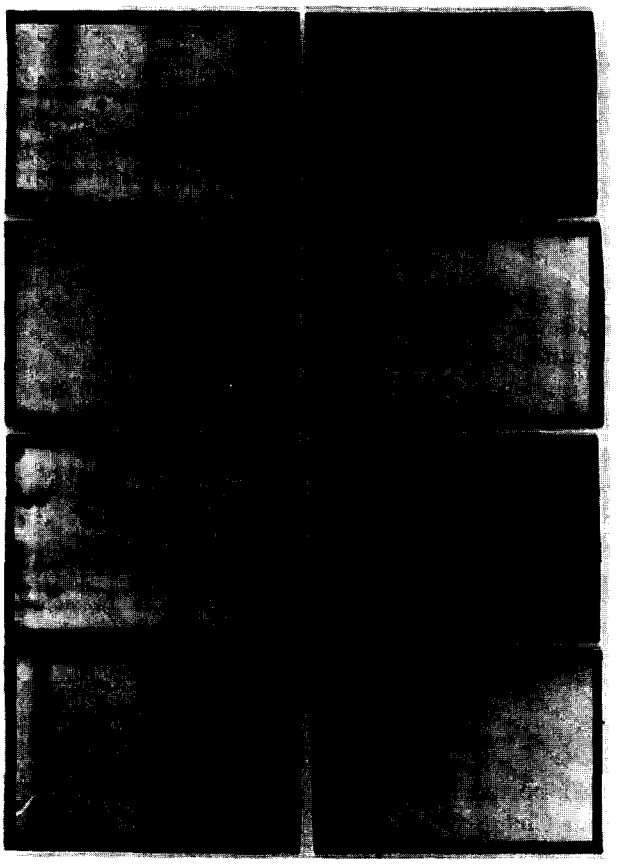

Fig. 9. Focal area of two-dimensional gel electrophoresis (molecular mass between 64 kDa to 50 kDa with a p*I* between 5.7 to 6.5) pattern of eight thyroid tissues with nodular hyperplasia.

We hope 2D gels will be used as a diagnostic tool to elucidate tumor specific proteins in the detection of well-differentiated thyroid cancers. From the 2D maps specific proteins with uniform molecular mass and p*I* can be detected. Using these data, we are able to purify proteins which can be used as tumor markers. From our limited data spots a1–a4 may be tumor markers for the human follicular thyroid neoplasm. When comparing the maps from normal tissues to nodular hyperplasia, follicular adenoma, and follicular carcinoma the protein spots in group "a" were more prominent. These protein spots may be stimulatory proteins or inhibitory proteins encoded by the tumor suppressor gene.

The genetic basis for malignant progression in the thyroid is still unclear. In cancer biology, it is important to know the pattern of progression from low to high malignancy in thyroid carcinoma. Like colon cancer, follicular thyroid cancer was thought to have a strictly linear progression [16]. The normal follicular cells progressed from adenoma to differentiated carcinoma to anaplastic carcinoma. From the previous review, oncogenesis in thyroid follicular cells was multistep and multiroute. The progression of normal thyroid tissues to follicular thyroid carcinoma was related to RAS and p53 oncogene mutation. The development of papillary thyroid carcinoma was related to RET and TRK oncogene mutation. Genetic alterations in a single amino acid can alter the charge of a peptide molecule. This change could be detected by 2D gel electrophoresis. In the present study the 2D

map from follicular adenoma was similar to that of follicular carcinoma but quite different from that of papillary carcinoma. These results demonstrated "linearity" of tumor progression [17].

4. Conclusions

In summary, the identified protein spots represented qualitative and quantitative changes of human thyroid tissues with our current level of staining. There were marked differences between the 2D maps of nodular hyperplasia and follicular thyroid carcinoma and normal thyroid tissue. Further studies may reveal the oncoproteins which are responsible for the transformation of thyrocytes from benign to malignant cells.

Acknowledgements

This investigation was supported by grant CMRP 355 to J.D. Lin from Chang Gung Memorial Hospital, Taipei, Taiwan. We thank Dr. Chau-Hsiung Chang for his interest and encouragement during this study.

References

[1] L.B. Woolner, O.H. Beahrs and B.M. Black, *Am. J. Surg.*, 102 (1961) 354.
[2] K. Shimaoka, J. Badillo and J.E. Sokal, *J. Am. Med. Assoc.*, 181 (1962) 179.
[3] J.B. Vander, E.A. Gaston and T.R. Dawber, *Ann. Intern. Med.*, 69 (1968) 537.
[4] J.M. Miller, J.I. Hamburger and S. Kini, *J. Am. Med. Assoc.*, 241 (1979) 481.
[5] M.E. Molitch, J.R. Beck and M. Dreisman, *Endocrine Rev.*, 5 (1984) 185.
[6] A.J. Van Herle, P. Rich and B.E. Ljung, *Ann. Intern. Med.*, 96 (1982) 221.
[7] H. Gharib, J.R. Goellner and A.R. Zinsmeister, *Ann. Intern. Med.*, 101 (1984) 25.
[8] S.M. Hanash and J.R. Strahler, *Nature*, 337 (1989) 485.
[9] H. Gulle, B. Schoel and S.H.E. Kaufmann, *J. Immunol. Methods*, 133 (1990) 253.
[10] N. Isoda, E. Kajii, S. Ikemoto and K. Kimura, *J. Chromatogr.*, 534 (1990) 47.
[11] E. Kajii, S. Iwamoto and S. Ikemoto, *J. Chromatogr.*, 533 (1990) 207.
[12] U. Bjorkman, R. Ekholm, L.E. Ericson and T. Ofverholm, *Mol. Cell Endocrinol.*, 5 (1976) 3.
[13] P.H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
[14] M.W. Ashcraft and A.J. Van Herle, *Am. J. Med.*, 71 (1981) 806.
[15] A.J. Van Herle, P.D. Rosenblit and T.L. Van Herle, *J. Endocrinol. Invest.*, 12 (1989) 171.
[16] N.R. Farid, Y. Shi and M. Zou, *Endocrine Rev.*, 15 (1994) 202.
[17] D. Wynford-Thomas, *Trends Endocrinol. Metab.*, 4 (1993) 224.