CANCER GENOMICS & PROTEOMICS *3*: 389-394 (2006)

Proteomic Studies of Galectin-3 Expression in Human Thyroid Diseases by Immunodetection

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Abstract. *Galectin-3 expression in thyroid diseases was studied by 1-DE immunoblotting. Expression was markedly elevated in thyroid papillary carcinoma, compared to follicular adenoma, follicular carcinoma or non-neoplastic diseases. Galectin-3 expression was also elevated in malignant cancers of bone, breast, colon, esophagus, larynx, lung and ovary. Four cases of thyroid papillary carcinoma with metastasis gave 2-3 bands on 1-DE immunoblotting. 2-DE immunoblotting of gelectin-3 showed 3 dark spots with MW/pI 32.9/8.29, 31.0/8.40 and 30.0/8.40 and 2 light spots.*

Thyroid cancer is a common endocrine malignancy, but pre-operative diagnosis can still pose problems. Thyroid scan, ultrasonography and fine-needle aspiration cytology (FNAC) are well established techniques for primary diagnosis of benign and malignant thyroid diseases (1), but misdiagnosis can occur, since it is sometimes difficult to distinguish the morphology of hyperplastic adenomatous nodules, well-differentiated follicular carcinomas and follicular variants of papillary carcinoma (2). Pre-operative diagnosis may be improved using strict protocols for obtaining specimens, with inclusion of clinical characteristics (3). However, reliable markers for early detection of malignant thyrocytes are required for accurate preoperative diagnosis of thyroid cancer. Immunohistochemical studies have shown high-molecular weight keratin (HMWK) and involucrin showed strong activity in papillary neoplasm (4). In addition, immunohistochemical studies of CD44v6 and galectin-3 showed that immunodetection of galectin-3 on thyroid cells is highly specific and sensitive for identifying malignant lesions of the thyroid (5).

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Key Words: Galectin-3, thyroid diseases, proteomics, immunodetection, papillary carcinoma.

The galectins are a family of animal β-galactoside-binding proteins, defined by their affinity for β-galactoside and their sequence homology in the carbohydrate-binding site (6). Galectin-3 (previously described as IgE binding-protein, CBP35, CBP30, Mac-2, L-29, L-31, L-34 and LBL) is a member of the endogeneous β-galactoside-binding proteins, which are expressed broadly in normal and neoplastic tissues. It is a 30 kDa monomeric protein, with known structure and genome sequence (7). Galectin-3 is believed to be able to bind with laminin, carcinoembryonic antigen and lysosome-associated membrane proteins (8, 9). However, the role of galectin-3 appears to depend on the subcellular localization. Thus, cell surface galectin-3 mediates cell-to-cell adhesion (10) and cell to extracellular matrix (ECM) interactions (11). Nuclear galectin-3 is involved in pre-mRNA splicing (12). Cytoplasmic galectin-3 is involved in apoptosis (13, 14), cellular proliferation (15) and differentiation (16). Immunohistochemical studies of human tumors, such as colorectal (17), thyroid (5), tongue (18), liver (19) and breast carcinomas (20) have suggested that the pattern of galectin-3 expression may serve as a tumor marker for prediction of metastasis, progression and invasion.

Proteomic studies in our laboratory have shown differences in protein expression in thyroid tissues with different diseases (21). Thus, cathepsin B is up-regulated in neoplastic thyroid tissues (21), while elevated levels of ATP synthase D chain and prohibitin in papillary carcinoma (PC) can distinguish it from follicular carcinoma (FC). However, we were not able to detect galectin-3 by two-dimensional gel electrophoresis (2-DE) with protein staining. On the other hand, immunohistocytochemical studies have suggested that galectin-3 may be a new marker for discriminating benign from malignant thyroid lesions (5), and other work on galectin-3 expression in thyroid diseases have been reported, also mainly *via* immunohistochemical techniques (22-25).

Materials and Methods

Sample preparation. Tumor tissues (s026h/48) were collected from the Pathology Department, Pramongkutklao Hospital, Bangkok and

Table I. *Medical diagnosis of patients with various thyroid diseases.*

Sample ID	Age/Gender	Diagnosis	Metastasis	Inflammation
T1	60/F	Adenomatous colloid goiter	NA	No
T2	47/F	Nodular colloid goiter	NA	Mild
T3	49/F	Adenomatous colloid goiter	NA	Mild
T4	24/F	Multinodular colloid goiter	NA	No
T5	47/F	Multinodular colloid goiter	NA	Mild
T6	27/F	Nodular colloid goiter	NA	Moderate
T7	48/F	Nodular colloid goiter	NA	No
T8	14/M	Diffuse thyroid hyperplasia	NA	Moderate
T9	21/F	Diffuse thyroid hyperplasia	NA	No
T10	21/F	Diffuse thyroid hyperplasia	NA	No
T11	12/M	Diffuse thyroid hyperplasia	NA	Mild
T12	17/F	Diffuse thyroid hyperplasia	NA	Moderate
T13	22/F	Diffuse thyroid hyperplasia	NA	Mild
T14	42/F	Diffuse thyroid hyperplasia	NA	Mild
T15	27/F	Diffuse thyroid hyperplasia	NA	Moderate
T16	34/F	Follicular adenoma	NA	Moderate
T17	47/F	Follicular adenoma	NA	Mild
T18	37/M	Follicular adenoma	NA	No
T19	32/F	Follicular adenoma	NA	No
T20	18/F	Follicular adenoma	NA	No
T21	42/M	Follicular adenoma	NA	No
T22	54/M	Follicular adenoma	NA	No
T23	30/F	Follicular adenoma	NA	Mild
T24	22/F	Follicular adenoma	NA	Mild
T25	28/F	Follicular adenoma	NA	No
T26	20/F	Follicular carcinoma, capsular	No	Mild
T27	48/F	Follicular carcinoma, capsular and vascular	No	No
T28	30/M	Follicular carcinoma, capsular and vascular	No	No
T29	32/F	Follicular carcinoma, capsular and vascular	No	Moderate
T30	51/M	Follicular carcinoma, capsular	No	Mild
T31	73/M	Papillary carcinoma, extrathyroid gland extension	No	No
T32	80/F	Papillary carcinoma	No	No
T33	23/F	Papillary carcinoma	Node metastasis	Mild
T34	70/F	Papillary carcinoma	Node metastasis	No
T35	33/M	Papillary carcinoma	Node metastasis	Mild
T36	36/F	Papillary carcinoma	No	No
T37	32/F	Papillary carcinoma	No	Mild
T38	66/M	Papillary carcinoma	Node metastasis	Mild

NA = not applicable.

stored at –70˚C until analysis. Adjacent tissue was formalin-fixed, paraffin-embedded and routinely examined for diagnosis. Tumors were characterized on the basis of size, lymph node status, histopathology, and graded according to standard criteria (WHO). Diagnosis of the 38 subjects studied, summarized in Table I, included goiter (G, 7 cases), diffuse hyperplasia or Graves' disease (Hy, 8 cases), follicular adenoma (FA, 10 cases), follicular carcinoma (FC, 5 cases) and papillary carcinoma (PC, 8 cases). Thyroid tissues were homogenized in distilled water containing 1 mM phenylmethanesulfonylfluoride, 10 μg/ml pepstatin A, 5 μg/ml bestatin, and centrifuged for 30 min at 12,000 rpm. Protein content of the supernatant was determined using the Bradford method (26).

SDS-PAGE immunodetection. Samples (20 μg protein) were mixed with sample buffer, boiled and applied to 12% T SDS polyacrylamide gels (100 x 80 x 0.75 mm) (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed in a Hoefer system (Hoefer, Inc., San Francisco, CA, USA) at 10 mA, room temperature for 1.5 h, followed by electroblotting of proteins from the gel onto nitrocellulose membranes (Hybond ECL; GE Healthcare, Buckinghamshire, UK) at 100 V for 30 min at 4˚C. After blocking in 5% non-fat dry milk, membranes were probed with 1:1000 diluted anti-galectin-3 monoclonal antibodies (Research Diagnostics, Concord, MA, USA), repeatedly washed in 20 mM Tris buffered-saline, pH 7.6, containing 0.1% Tween 20, and then incubated in 1:5000 rabbit anti-mouse immunoglobulin G (IgG; Dako Cytomation, Glostrup, Denmark) for 1 h. After washing, the reaction was developed using the ECL plus detection system, with high-performance film (Hyper-film ECL; GE Healthcare).

2-DE immunoblotting. This was performed with selected colon and thyroid PC samples. Tissues were homogenized in lysis buffer (9 M urea, 2% CHAPS, 2% DTT, 2% Ampholine pH 3.5-10, 1 mM PMSF, 10 μg/ml pepstatin A, 5 μg/ml bestatin) and centrifuged for 30 min at 12,000 rpm. 2-DE was performed using first-dimension isoelectric focusing (IEF) on 70 mm nonlinear pH 3-10 or 6-11, IPG gel strips

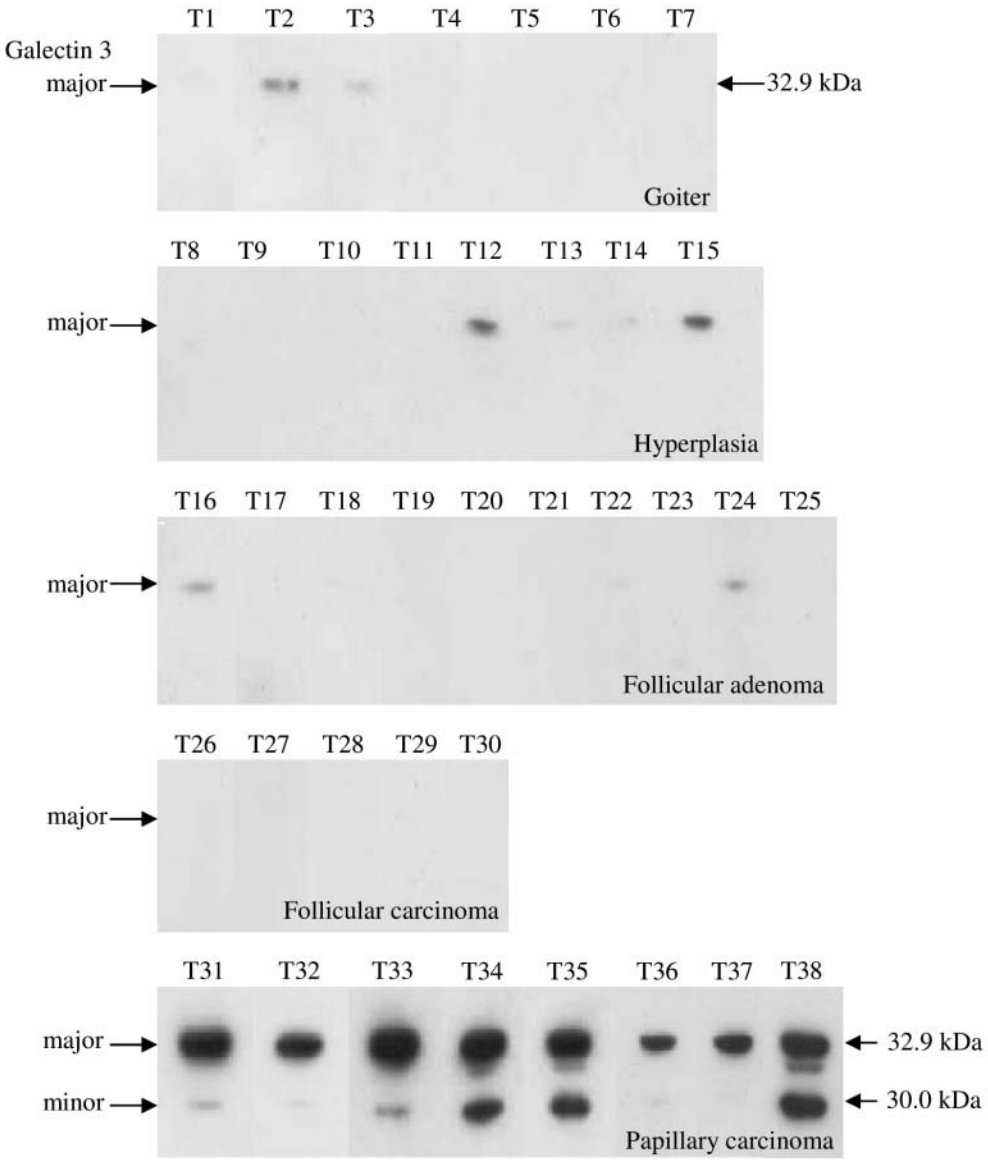

Figure 1. *The SDS-PAGE blotting of 38 cases of various thyroid diseases including goiter (T1-T7), hyperplasia (T8-T15), follicular adenoma (T16-T25), follicular carcinoma (T26-T30) and papillary carcinoma (T31-T38) were stained with anti-galectin-3 antibody and detected by ECL Western blot detection reagent. The major band of MW 32.9 and minor band of 30.0 kDa represented galectin-3.*

(GE Healthcare) at 6,500 Vh in a Pharmacia LKB Multiphor II system (GE Healthcare), followed by second-dimension on 14% T SDS polyacrylamide gels (100 x 80 x 1.5 mm) in a Hoefer system at 20 mA, room temperature for 2 h. After electrophoresis, proteins were transferred to nitrocellulose membrane by Western blotting, followed by immunodetection as described above.

Results

The expression of galectin-3 in thyroid tissues from Thai patients with various diseases by SDS-PAGE immunodetection was investigated to see whether its expression is related to cancer. The SDS-immunoblotting for galectin-3 in thyroid tissues from the 38 cases of thyroid disease gave the results shown in Figure 1. PC (T31-T38) showed very high levels of expression, distinguishing it from other thyroid diseases. In addition, multiple galectin-3 bands were often detected: of the 8 PC cases, 2 cases showed 2 bands (T31, T33), 3 cases showed 3 bands (T34, T35, T38), and 3 cases showed a single band (T32, T36, T37) of galectin-3. The four PC cases showing metastasis (T33, T34, T35, T38) showed particularly intense main bands at 32.9 kDa, together with minor bands.

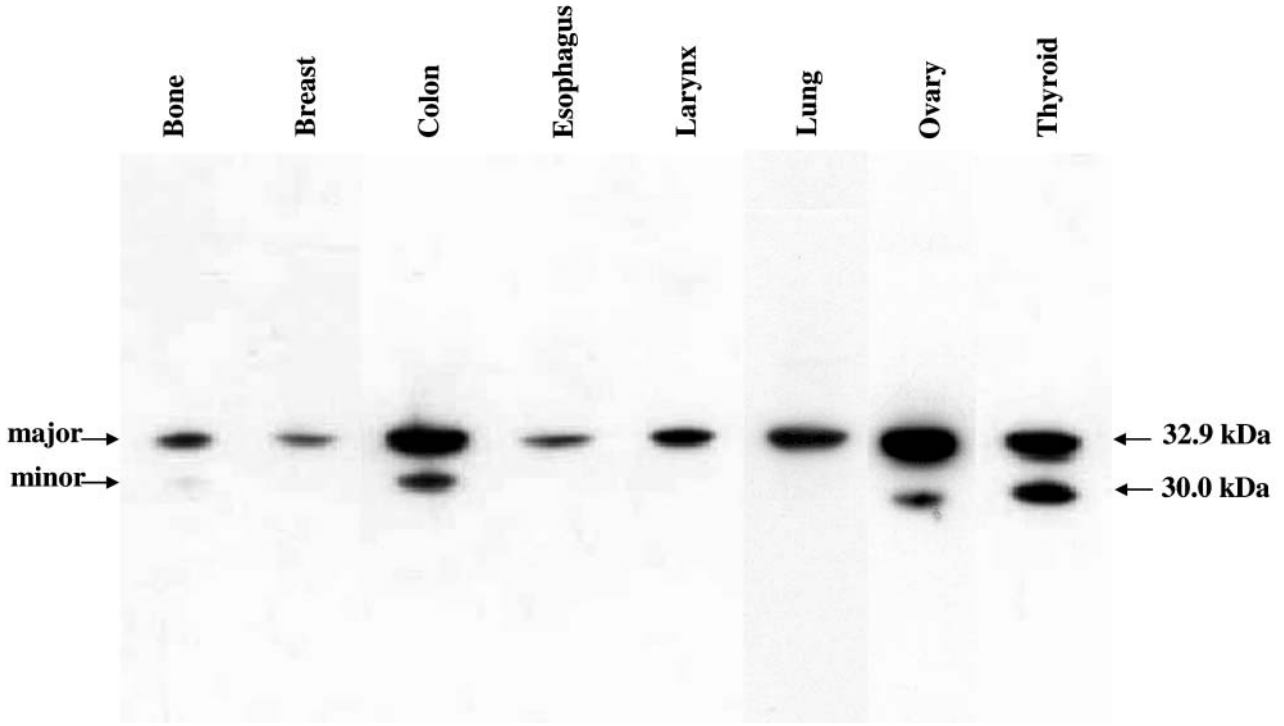

Figure 2. *The comparison of 1-DE immunodetection of galectin-3 from various cancer tissues. Results show representative data on galectin-3 expression in bone, breast, colon, esophagus, larynx, lung, ovary and thyroid cancer tissues. Twenty-five μg protein was loaded in each case.*

On the other hand, thyroid tissues from other diseases showed little or no galectin-3 expression (Figure 1). Thus, no immunoreactivity for galectin-3 could be detected in five out of seven G cases, six out of eight Hy cases, eight out of ten FA and all FC cases. Low levels of galectin-3 expression could be detected in some cases of G (T2, T3), Hy (T12, T15) and FA (T16, T24), but all these cases were also associated with inflammation, as summarized in Table I.

Galectin-3 expression in other malignant cancer tissues was also studied by 1-DE immunodetection. These included cancer of the bone (3 cases), breast (3), colon (7), esophagus (3), larynx (2), lung (3) and ovary (3). Representative results of galectin-3 expression in various tissues are shown in Figure 2, while diagnostic information of the samples shown are listed in Table II. Galectin-3 expression is found in all cases (Figure 2), but particularly high levels are found in tissues from colon (C42), ovarian (O46), and thyroid PC (T38) cancer. Notably multiple bands were also found in the thyroid, ovarian and colon specimens (Figure 2), similar to that found in the majority of the PC cases shown in Figure 1.

The proteomic patterns of (PC) thyroid (T38) and a representative colon cancer tissue (C42) are shown in Figure 3. The expression of galectin-3 in colon and thyroid showed similar patterns. Colon tissue (C42) showed six dark spots, with MW/pI 32.9/8.29 (spot 1), 32.9/8.35 (spot 6), 32.0/8.40 (spot 2), 32.9/8.20 (spot 3) 30.0/8.20 (spot 4) and 30.0/8.40 (spot 5), while thyroid PC (T38) showed 3 dark spots (spots 1, 2, 5) and 2 light spots (spots 3, 4).

Discussion

In the present studies, 1-DE SDS-immunoblotting showed that galectin-3 was expressed at very high levels in PC of the thyroid, while other diseases of the thyroid generally did not show galectin-3 expression, unless associated with inflammation, where low levels of expression were detectable. The high levels of galectin-3 expression found here in PC by 1-DE immunoblotting agree with previous studies via immunohistochemistry, which also showed that galectin-3 appears to be a reliable indicator for diagnosis of PC (22, 24). This finding may be related to the observation that stromal elastosis also appears to be a histopathological indicator for dignosis of PC (27). These observations led to the idea that elastokines may play a role in regulating tumor progression and metastasis through the activation of elastin receptor (S-Gal) cascades. Since galectin-3 contains GXXPG sequence repeats like tropoelastin and S-Gal, it has been suggested that galectin-3 may bind to S-Gal, and thereby stimulate elastogenesis and tumor progression (28).

Our studies also show that galectin-3 expression was markedly increased in extrathyroid gland extension (T31) and in metastasis (T33, T34, T35, T38). In addition, faster moving

Table II. *Medical diagnosis of cancer specimens from different tissues.*

Sample ID	Age/Gender	Diagnosis	Metastasis
Bo40	16/F	Osteosarcoma (Bone)	No data
Br41	68/F	Infiltrating ductal carcinoma, grade III (Breast)	No
C42	63/M	Adenocarcinoma, grade II (Colon)	No
E43	56/M	Squamous cell carcinoma, moderately differentiated (Esophagus)	No
La44	47/M	Squamous cell carcinoma, grade II (Larynx)	Metastasis
Lu45	49/M	Adenocarcinoma, poorly differentiated (Lung)	Metastasis
O46	18/F	Mucinous papillary cystadenocarcinoma (Ovary)	No

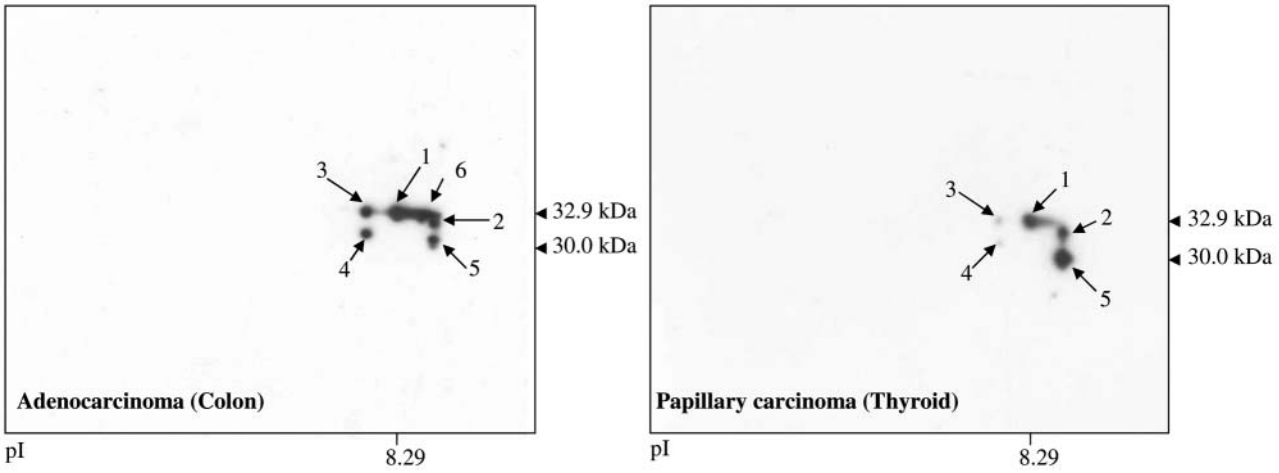

Figure 3. *Two-dimensional immunodetection of galectin-3 in adenocarcinoma (colon) and papillary carcinoma (thyroid). Colon showed 6 dark spots with pI/MW 32.9/8.29 (spot 1), 32.0/8.40 (spot 2), 32.9//8.20 (spot 3), 30.0/8.20 (spot 4), 30.0/8.35 (spot 6) while PC showed 3 dark spots (spots 1, 2, 5) and 2 light spots (spots 3, 4). One hundred μg protein was loaded in both cases.*

band(s) were evident in specimens with metastasis, being particularly intense in specimens T34, T35, T38. This marked increase in galectin-3 expression found here is in agreement with the work of Kawachi *et al.* (25), who showed that galectin-3 expression was significantly higher in primary lesions of papillary carcinoma with lymphatic metastases compared to primary lesions without metastases. Indicators of metastasis or potential metastasis would be of much use for diagnosis, but further studies will be needed, to show whether the greater intensity of the immunoblot bands and/or the presence of extra bands will be useful as such indicators.

1-DE immunodetection also showed galectin-3 expression in other malignant cancer tissues from bone, breast, colon, esophagus, larynx, lung, and ovary (Figure 2). Multiple bands were only present in thyroid, colon and ovary cancer tissues, but these samples did not show metastasis. Since, there have been no previous reports of multiple bands of galectin-3, we further studied the expression of galectin-3 from thyroid PC and colon by 2-DE immunodetection. Thyroid PC showed 3 dark spots with 32.9/8.29 (spot 1), 32.0/8.40 (spot 2) and 30.0/8.40 (spot 5), plus 2 light spots with 32.9/8.20 (spot 3) 30.0/8.20 (spot 4). At least some of these spots are likely to arise from post-translational changes, such as phosphorylation of galectin-3, which has been shown to occur in other systems (29, 30). In any event, one of the dark spots (spot 5) appears to be found mainly in the specimens with metastasis (T33, T34, T35, T38), and is not likely due to phosphorylation alone, since it has a decreased MW 30.0 kDa, compared to the main 32.9 kDa spot.

Conclusion

1-DE immunoblotting for galectin-3 allows thyroid PC to be readily distinguished from FA and FC, as well as from non-neoplastic diseases. This will be useful for improving diagnosis of PC, since there are many variant forms of PC, including those containing follicular structures. In addition, multiple bands may be found in metastasis. However, inflammation can result in weak bands of galectin-3 being occasionally found in other thyroid diseases, but these tend to be much fainter. Galectin-3 was also detected in other malignant cancer tissues by immunoblotting, especially in colon and ovarian cancer. The MW/pI of the multiple forms of galectin-3 in thyroid PC and colon were also

characterized by 2-DE immunoblotting for the first time. However, further studies are necessary to determine whether these are due to post-translational modification, and if so, of what kind.

Acknowledgements

This investigation was supported by the Chulabhorn Research Institute. Jisnuson Svasti is a Senior Scholar of the Thailand Research Fund.

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Received August 30, 2006
Revised October 12, 2006
Accepted October 17, 2006