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Evaluation of a new method for the diagnosis of alterations of *Lens culinaris* agglutinin binding of thyroglobulin molecules in thyroid carcinoma

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Abstract

Background: The measurement of serum thyroglobulin (Tg) is widely used as a marker for recurrence of thyroid carcinoma following total thyroidectomy. However, this method cannot differentiate between benign and malignant disease. We focused on the sugar chain in the Tg molecule and investigated the usefulness of *Lens culinaris* agglutinin (LCA)-reactive Tg ratios in sera and wash fluids obtained during fine-needle aspiration (FNA) for the detection of thyroid carcinoma.
Methods: The study was performed using 203 serum samples (115 from patients with benign thyroid disease and 88 from patients with thyroid carcinomas) and 176 wash fluid samples (143 benign, 21 malignant, and 12 inconclusive). LCA-reactive Tg ratios were determined using an enzyme-linked immunosorbent assay, and a comparison was made between malignant and benign lesions.
Results: In serum, the ratio in patients with malignancy was 79.5±6.0 [mean±standard deviation (SD)], significantly lower than in patients with benign lesions (84.9±3.5). The ratios in wash fluid from malignant lesions (75.8±18.9) were also significantly lower than those from benign lesions (85.6±3.9).
Conclusions: These results suggest that this method could distinguish between benign and malignant lesions and may be useful for screening serum and wash samples.
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Introduction

Measurement of serum thyroglobulin (Tg), thyroid ultrasonography and fine-needle aspiration (FNA) cytology are widely used diagnostic methods to detect thyroid carcinoma (1, 2). Although measurement of the serum Tg concentration is suitable for screening to detect recurrence or for follow-up following surgery for carcinoma surgery (3–6), Tg concentrations may be increased even in benign thyroid diseases. The current method cannot differentiate between benign and malignant disease. Thus, measurement of serum Tg concentrations is not suitable for screening to detect primary thyroid carcinoma (7–9). Even with use of ultrasonography, cases showing atypical findings cannot be diagnosed definitively (10, 11). In addition, some cases may be difficult to differentiate, even with use of FNA cytology due to insufficient amounts of sample (12–14). Differentiation between thyroid follicular carcinoma and follicular adenoma is difficult, even with use of cytology (15–17).

Although a method for a differential diagnosis of malignancy based on the identification of specific genes has been reported (18, 19), it has not yet been applied clinically. It is difficult to differentiate benign and malignant tumors based on Tg concentrations, and there are few methods for the management of large numbers of these types of samples in the laboratory (20).

In the present study, we focused on the occurrence of changes in the sugar chain in the Tg molecule during the transformation of thyroid cells into cancer cells. We developed a new method using enzyme-linked immunosorbent assay (ELISA) to identify differences in the sugar chain by reactivity with lectin. We investigated whether this method could be used as a screening method for primary thyroid carcinoma, using a large number of serum or wash fluid samples obtained during FNA.

Materials and methods

Clinical materials

This study, as well as the use of all clinical materials described below, was approved by the individual Institutional Ethical Committees. The investigation conformed to the

guidelines for the handling of animals from the Research Committees of Kagawa Prefectural College.

This study was performed using 49 thyroid tissue samples (18 cases of papillary carcinoma, 6 follicular carcinoma, 11 benign follicular adenoma, 7 adenomatous goiter, 1 anaplastic carcinoma, and 6 Graves disease), obtained during surgery at the Department of Surgery II of Shinshu University, and 203 serum samples (115 from patients with benign thyroid disease, 88 from patients with thyroid carcinoma including 83 with papillary carcinoma and 5 with follicular carcinoma). We also used 176 wash fluid samples (143 diagnosed as benign, 21 malignant, and 12 inconclusive) obtained during FNA from the same department and from Azumi General Hospital which is associated with Shinshu University. To obtain wash fluid, we performed aspiration of cells under ultrasonographic guidance. After the cells were placed onto slides, the wash fluid from the puncture needle was used. Tissue samples were cryopreserved until use. Informed consent was obtained from all patients.

Confirmation of changes in Tg sugar chains in thyroid tissues

Tissue samples from 18 patients with papillary carcinoma, six with follicular carcinoma, one with anaplastic carcinoma, 11 from patients with benign follicular adenoma, seven with adenomatous goiter, and from six patients with Graves disease were soaked in 0.1 mol/L phosphate-buffered saline (pH 7.2, 1 μmol/L phenylmethylsulfonyl fluoride). The samples were homogenized and centrifuged at 10,000×*g* for 30 min. The supernatant was separated with an ACA34 column to obtain a crude solution of Tg. The optical density of the protein concentration was measured at 280 nm.

The Tg was isolated with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane and allowed to react with labeled *Lens culinaris* agglutinin (LCA)-horseradish peroxidase (HRP) conjugate, HRP-LCA (J-OIL MILLS, Kanagawa, Japan) to investigate whether there was any difference in the affinity of Tg derived from each tissue sample.

Reaction with anti-Tg monoclonal antibody (TgMAb) was performed to confirm that the isolate was indeed Tg. We also used an alternate method to investigate abnormalities in Tg sugar chains in carcinomas and benign tumors. Tg from each tissue was isolated with use of V8 protease (*Staphylococcus aureus* V8; Sigma, St. Louis, MO, USA), subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and allowed to react with labeled lectin or labeled TgMAb.

Preparation of TgMAb

Using Tg obtained from Graves disease tissues as an antigen, a solution mixed with Freund's complete adjuvant (Wako Pure Chemical Industries, Osaka, Japan) at a ratio of 1:9 was administered intraperitoneally to BALB/c mice (Charles River Laboratories Japan, Inc., Shiga, Japan) at a dose of 80 μg/mouse. After confirmation of an increase in anti-Tg antibody titers, cell fusion between mouse spleen lymphocytes and P3U1 cells (Wako Pure Chemical Industries, Osaka, Japan) was performed using the method described previously to obtain a target clone. The antibody titer was confirmed by ELISA using labeled antibody isolated in our laboratory. The TgMAb that was produced was reacted with standard Tg solution to confirm recognition of Tg.

Measurement of LCA-reactive Tg ratio in serum

Measurement of LCA-reactive Tg ratio was performed using ELISA, as shown in Figure 1. LCA reacts with normal Tg (N-Tg). Thus, HRP-labeled TgMAb cannot react with Tg-LCA complex. However, LCA cannot react with Tg from malignant tissue (M-Tg); HRP-labeled TgMAb reacts with M-Tg.

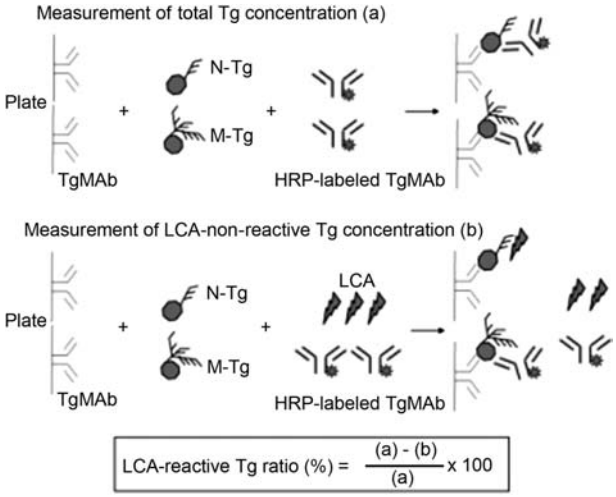

Figure 1 The theory of measurement method of LCA-reactive Tg ratio, using ELISA.
This method used the property of LCA lectin that inhibits anti-Tg monoclonal antibody reaction. Total Tg concentration and LCA-non-reactive Tg concentration were measured, and the LCA-reactive Tg ratio (%) was calculated. N-Tg, normal Tg; M-Tg, Tg from malignant tissue.

To measure the LCA-reactive Tg ratio in the 203 serum samples from patients with thyroid disease (benign in 115 cases, malignant in 88) using ELISA, we coated immunoplates with TgMAb isolated in our laboratory (Nalge Nunc International K.K., Roskilde, Denmark). Aliquots of 50 μL from each serum sample were added to these plates and allowed to react at room temperature for 1 h. These were then washed and 100 μL of LCA lectin (J-OIL MILLS, Kanagawa, Japan) diluted 1000-fold with buffer, or buffer alone as a negative control, was added.

The mixtures were allowed to react overnight at 37°C. They were then washed and 100 μL of HRP-labeled anti-TgMAb solution (prepared by the method described above) was added, allowed to react for 1 h, developed with 100 μL of substrate [3,3′,5,5′-tetramethylbenzidine (TMB); Kierkegaard & Perry Laboratories, Inc, Gaithersburg, MD, USA], and measured using a microplate reader. The LCA-reactive Tg ratio was calculated according to the following equation:

$$\text{LCA-reactive Tg ratio (\%)} = \frac{(\text{Total Tg concentration} - \text{LCA-non-reactive Tg concentration})}{\text{Total Tg concentration}} \times 100$$

For comparison, Tg concentrations in all serum samples were measured by the current method.

Measurement of LCA-reactive Tg ratio in wash fluid

Using the 176 samples of wash fluid (benign in 143 cases, malignant in 21, inconclusive in 12), measurements were performed using ELISA, in a manner similar to that described for serum, to determine the LCA-reactive Tg ratio (%).

FNAs of tumor were performed at two different times in the same patient from 18 randomly selected cases to determine the reproducibility of the LCA-reactive Tg ratio. The

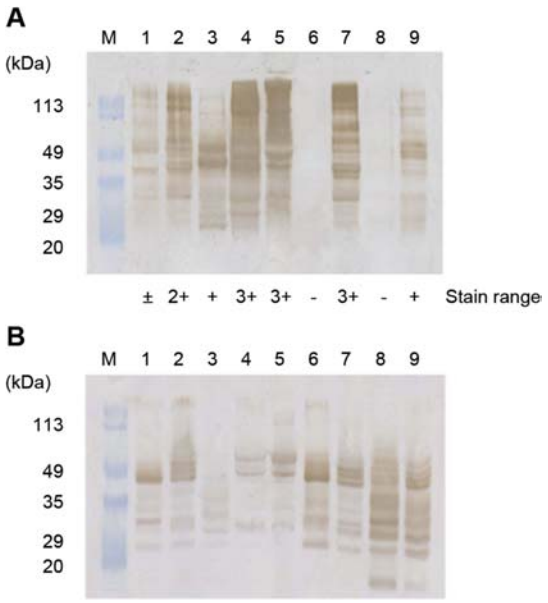

Figure 2 Immunoblotting of crude Tg solution.
(A) LCA-lectin blotting; (B) TgAb blotting. The range of – indicates no staining; ± indicates slight staining; + indicates weak staining; 2+ indicates moderately strong staining; and 3+ indicates strong staining. M, marker; Nos. 1, 6: papillary carcinoma; Nos. 2, 8, and 9: follicular carcinoma; No. 3: Graves disease; No. 4: normal; No. 5: adenomatous goiter; No. 7: follicular adenoma.

time period between the first and second FNA was about 1 month.

Statistical methods

The significance of differences was examined using Student's t-test. $p<0.01$ were considered to be statistically significant.

Results

Alteration of Tg sugar chain in thyroid tissues

We used four types of lectins (LCA, concanavalin A, ricinus communis agglutinin-120, and datura stramonium agglutinin) to investigate which types of lectins could detect a significant difference between benign and malignant disease. LCA binding to Tg in malignant disease was significantly lower than in benign disease (data not shown). Thus, we used this lectin.

Immunoblotting of crude Tg solution and labeled lectin LCA in each thyroid tissue specimen demonstrated strong staining for Tg sugar chains in the group with benign tumors and those with Graves disease. Weak staining was observed in patients with papillary carcinoma.

After Tg was digested with use of V8 protease, immunoblotting was performed as described above. Carcinoma tissue showed diverse patterns, from weak to slightly stronger bands with LCA staining. All tissues from those with benign tumors and Graves tissues showed strong bands (Figure 2A).

In Figure 2A, M means marker; Nos. 1 and 6 are papillary carcinomas; Nos. 2, 8, and 9 are follicular carcinomas; No. 3 is Graves' disease; No. 4 is normal; No. 5 is adenomatous goiter; and No. 7 is follicular adenoma. The benign group tended to show strong staining, and the malignant group tended to show weak staining.

Figure 2B showed anti-Tg antibody (TgAb) blotting. Nos. 6 and 8 showed strong staining in the TgAb blot and no staining in LCA-lectin blot. Thus, there was an apparent difference in immunoblotting results of the sugar chains in Tg molecules in tissues from patients with carcinoma and those with benign tumors.

Table 1 shows a summary of the immunoblotting results. The group with malignant disease showed staining in the range of – to 2+, whereas the benign group showed strong staining in the range of + to 3+. The range of – indicates no staining, examples include Nos. 6 and 8 in Figure 2A; ±means slight staining, such as No. 1 in Figure 2A; + indicates weak staining, examples include Nos. 3 and 9 in Figure 2A; 2+ indicates moderately strong staining, such as No. 2 in Figure 2A; and 3+ indicates strong staining, such as Nos. 4, 5, and 7 in Figure 2A.

Measurement of LCA-reactive Tg ratio in serum

Samples from patients with benign (115 cases) and malignant disease (88 cases) were examined individually to determine the LCA-reactive Tg ratio in serum using ELISA.

Table 1 Summary of immunoblotting results.

Clinical diagnosis	Histological type	Stain pattern					
		n	–	±	+	2+	3+
Benign	Graves disease	6	–	–	1	–	5
	Follicular adenoma	11	–	–	–	1	10
	Adenomatous goiter	7	–	–	–	1	6
Malignant	Papillary carcinoma	18	3	6	9	–	–
	Follicular carcinoma	6	1	1	3	1	–
	Anaplastic carcinoma	1	1	–	–	–	–

Staining pattern of crude Tg solution reacting with labeled lectin was classified as – to 3+. The carcinoma group showed staining in the range of – to 2+, whereas the benign group showed strong staining in the range of + to 3+. The range of – indicates no staining; ± indicates slight staining; + indicates weak staining; 2+ indicates moderately strong staining; and 3+ indicates strong staining.

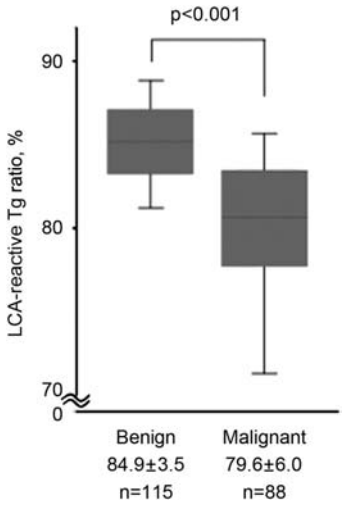

Figure 3 Distribution of LCA-reactive Tg ratios in sera.
The LCA-reactive Tg ratio in benign cases was 84.9±3.5 (mean±SD), and in malignant cases was 79.5±6.0 (mean±SD).

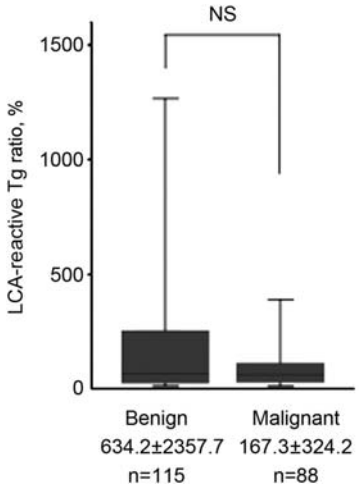

Figure 4 Total Tg concentration in sera.
Tg concentration in sera was measured using the current method. There was no significant difference between the benign and the malignant group.

In comparison with the benign group, the malignant group showed a significantly lower ratio of LCA-reactive Tg [benign: mean±standard deviation (SD); 84.9±3.5, malignant: mean±SD; 79.5±6.0, $p<0.001$] (Figure 3).

When an LCA-reactive Tg ratio of 81.8% was used as the cut-off threshold, with patients below this level defined as positive, the rate of positivity was 11.3% in the benign group and 61.4% in the malignant group (Table 2).

For comparison, Tg concentrations in serum samples were measured by the current method. There was no significant difference between the malignant group and the benign group (Figure 4).

Measurement of LCA-reactive Tg ratio in wash fluid

Three groups of wash fluid samples (143 benign, 21 malignant, and 12 inconclusive) were examined individually to determine the LCA-reactive Tg ratio using ELISA.

As shown in Figure 5, in comparison with the benign group, the malignant group showed a significantly lower ratio of LCA-reactive Tg (benign: mean±SD; 85.6±3.9; malignant: mean±SD; 75.8±18.9, $p<0.001$). Comparing the benign and inconclusive groups, the latter showed a significantly lower ratio (inconclusive: mean±SD; 78.0±19.4, $p<0.001$). However, there was no significant difference between the groups with malignant and inconclusive disease.

When an LCA-reactive Tg ratio of 80% was used as the cut-off threshold, with cases below this level defined as positive, the rate of positivity was 42.9% in the malignant group, while 93.7% of patients in the benign group were negative (Table 3).

Inconclusive cases, such as those with follicular tumor, tended to demonstrate low ratios of LCA-reactive Tg. This finding indicated that follicular carcinoma cases, which cannot be diagnosed by ultrasonography and cytological diagnosis, may be included in this group.

Reproducibility of LCA-reactive Tg ratio by multiple FNAs

In the 18 cases that underwent FNA of the tumor at two different times, approximately similar levels were shown on the two tests (Table 4). Reproducibility of the examination was thus confirmed.

Discussion

It has been reported that total Tg concentration in serum cannot distinguish between benign and malignant thyroid disease. We used LCA lectin and examined the variations in the sugar chains of Tg from sera and wash fluids. We confirmed that the LCA-reactive Tg ratio could distinguish between thyroid carcinoma and benign thyroid tumor.

Tg is a protein required for synthesis of thyroid hormone and is expressed specifically in the thyroid.

Table 2 Relationship between the LCA-reactive Tg ratio in sera and clinical diagnosis.

Clinical diagnosis	n	Positive	%	Negative	%
Benign	115	13	11.3	102	88.7
Malignant	88	54	61.4	34	38.6

When an LCA-reactive Tg ratio of 81.8% was used as the cut-off threshold, with cases below this level defined as positive, the positive rate tended to be higher in the malignant group (61.4%) than in the benign group (11.3%).

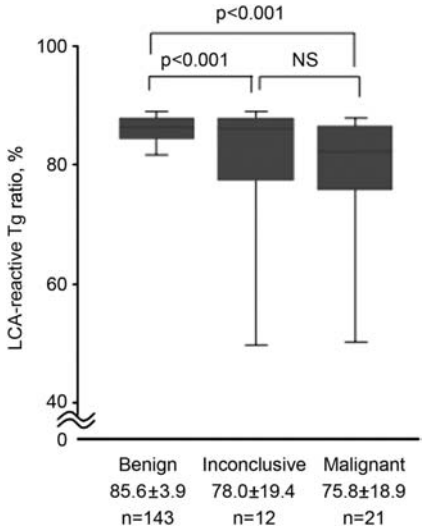

Figure 5 Distribution of LCA-reactive Tg ratios in washout fluids.
The LCA-reactive Tg ratio in benign lesions was 85.6± 3.9 (mean±SD), and in malignant lesions was 75.8±18.9 (mean±SD). In inconclusive cases, the ratio was 78.0± 19.4 (mean±SD).

Table 4 Reproducibility of LCA-reactive Tg ratio by multiple FNAs.

Case	LCA-reactive Tg ratio		
	First, %	Second, %	Difference
1	88.9	88.4	0.5
2	85.2	85.2	0
3	12.8	19.9	7.1
4	87.3	83.5	3.8
5	85.6	85.5	0.1
6	85.4	88.1	2.7
7	86.4	88.5	2.1
8	87.3	86.6	0.7
9	85.7	82.9	2.8
10	88.9	87.2	1.7
11	88.0	86.4	1.6
12	86.3	85.9	0.4
13	87.1	87.8	0.7
14	88.3	88.2	0.1
15	82.2	86.1	3.9
16	86.5	85.3	1.2
17	85.3	89.2	3.9
18	81.0	81.9	0.9

FNA was performed twice on the same tumor in 18 cases. These cases showed approximately the same levels on repeat examination, confirming the reproducibility of the examination.

It consists of two subunits with a molecular weight of 330,000. Tg is also known to contain 10% sugar chains (21–23). The sugar chains change in parallel with malignant alterations in the cells. In particular, the structure of the sugar chain of Tg produced by carcinoma cells changes considerably (24). Although there are two branches in normal cells, three to five branches are seen in carcinoma. In carcinoma, the sugar type is also known to change from a high mannose-dominant type to a mixed or Gal-GlcNac type (25). This study suggests that LCA reactivity could distingish between these variations in the sugar chain. LCA can react with N-Tg sugar chains from benign lesions, but not with varied sugar chains of Tg from patients with thyroid carcinoma. Moreover, the influence of autoantibody, detected in patients with chronic thyroiditis, is small because this method utilized the ratio.

Although lectin blotting of LCA showed a dramatic change between benign disease and malignancy, the data from ELISA using LCA was not dramatically changed. The reason for the discrepancy is presumed to be as follows. Lectin blotting of LCA was used for tumor tissues obtained during surgery. Thus, the data reflect the alterations in Tg sugar chains from tumor lesions only. Lectin blotting of LCA showed a dramatic change between benign disease and malignancy. However, ELISA with LCA used sera and wash fluids obtained during FNA. The serum contains Tg from benign or malignant lesions and from normal thyroid tissue. The data showed a ratio of Tg from lesion to total Tg; thus, it was not different. Wash fluid may also contain N-Tg because the aspiration needle passes through normal thyroid tissue.

We consider that the LCA-reactive Tg ratio has possible utility as a specific marker of thyroid carcinoma. This method has the following advantages:

1. Both the serum and wash fluid can be used as samples.
2. A differential diagnosis between malignant and benign lesions can be expressed as a numerical value, and does not rely on individual evaluations. Consequently, this allows for a more objective evaluation.
3. A great many cases can be examined simultaneously, making this method available for screening examinations.
4. This method is effective for cases where ultrasonography and cytological diagnosis are inconclusive.

However, diagnosis is difficult if the amount of Tg collected is small (5 ng/mL or less).

In conclusion, we developed a new method for the diagnosis of alterations in the sugar chains of Tg molecules, using the reactive ratio of LCA lectin. This method may be useful for distinguishing between

Table 3 Relationship between LCA-reactive Tg ratio in wash fluid and clinical diagnosis.

Clinical diagnosis	n	Positive	%	Negative	%
Benign	143	9	6.3	134	93.7
Malignant	21	9	42.9	12	57.1

When an LCA-reactive Tg ratio of 80% was used as the cut-off threshold, with cases below this level defined as positive, the positive rate tended to be higher in the malignant group (42.9%) than in the benign group (6.3%).

thyroid carcinoma and benign thyroid tumor. Either serum or wash fluid may be used in this method for the screening of primary thyroid carcinoma.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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