

Clinical Utility of an Automated Immunochemiluminometric Thyroglobulin Assay in Differentiated Thyroid Carcinoma

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Background: Thyroglobulin (Tg) measurements are important in the follow-up of patients with differentiated thyroid carcinoma (DTC). We evaluated the analytical and clinical performance of a new automated immunochemiluminometric assay for Tg (Tg-ICMA; Nichols Advantage Tg; Nichols Institute Diagnostics).

Methods: We used the Tg-ICMA to measure Tg concentrations in serum samples from 110 Tg antibody-negative DTC patients undergoing thyroid-hormone suppression therapy. Disease state at the time of measurement was assessed on the basis of routine follow-up data. We compared the clinical performance of this assay with the routinely used IRMA (ELSA-hTG; CIS Bio International).

Results: The detection limit and functional sensitivity of the Tg-ICMA, based on direct calibration to CRM-457, were 0.05 and 0.6 $\mu\text{g/L}$, respectively. No Tg-IRMA-positive cases were missed by the Tg-ICMA. Tg was measurable by Tg-ICMA (0.6–8.6 $\mu\text{g/L}$) but undetectable by Tg-IRMA (<1.5 $\mu\text{g/L}$) in 12 patients (11%). Clinical data showed evidence of disease in 4 of 12 patients (33%).

Conclusions: The Tg-ICMA is a sensitive and reproducible assay for identifying patients in follow-up for DTC with evidence of disease, but uncertainty remains with

regard to interpreting findings of measurable serum Tg in patients with no evidence of disease. Follow-up data are required to determine the predictive value of these isolated Tg results. New concepts, i.e., serial Tg measurements and risk stratification of patients, need to be tested to confirm the applicability of this assay for clinical practice.

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High-quality thyroglobulin (Tg)⁵ assays are needed because of the fundamental role of Tg measurements in the postoperative monitoring of patients with differentiated thyroid carcinoma (DTC). Tg is a very large and heterogeneous glycoprotein that serves as the prohormone for thyroid hormone synthesis. Tg is used as a tumor marker because thyroid cells are the only source of Tg in the human body (1). Thus, the presence of Tg after total thyroidectomy and ablative I-131 therapy indicates persistence or recurrence of DTC. In particular, increasing serum Tg concentrations are an early and reliable indicator of recurrent disease (2).

Several Tg assays have been developed, but these assays are prone to methodologic problems, such as differences in standardization, suboptimal assay sensitivity and interassay precision, hook effects, and interference attributable to Tg antibodies (TgAbs) (2). A lack of standardization can lead to difficulties with intermethod comparison of Tg results. Tg methods can also be too insensitive for monitoring DTC patients for disease recurrence. Poor interassay precision can make it impossible to reliably detect small changes in tumor size. Furthermore, the hook effect in sera with very high Tg concentrations can lead to falsely low Tg values. Interference by TgAbs leads to over- or underestimation of Tg concentrations, depend-

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⁵ Nonstandard abbreviations: Tg, thyroglobulin; DTC, differentiated thyroid carcinoma; TgAb, thyroglobulin antibody; ICMA, immunochemiluminometric assay; HAMA, heterophilic antibody; HBT, heterophilic blocking tube; and TSH, thyrotropin.

ing on the method used (2, 3). No current Tg method is devoid of TgAb interference in every patient (4).

Recently, fully automated chemiluminescence assays for Tg have been developed that use monoclonal antibodies specific for human Tg. These assays combine high sensitivity with short turnaround times (5, 6). We evaluated the analytical and clinical performance of a new automated immunochemiluminometric assay for Tg (Tg-ICMA).

Materials and Methods

PATIENTS

Since 1978, the Department of Endocrinology of the University Medical Centre Groningen has treated and followed ~600 patients with DTC. The present study included all 131 consecutive patients who visited our outpatient clinic for follow-up during the period of May to September 2003.

All patients [mean (SD) age, 54 (17) years; 22% male; median follow-up, 8 years (interquartile range, 2–18 years); see Table 1] previously underwent treatment with total thyroidectomy and lymph node dissection, if indicated, followed by I-131 treatment for ablation and, if necessary, for treatment of persistent or recurrent disease when I-131 uptake persisted [as described by Haveman et al. (7)]. Initial tumor staging was performed according to the postoperative tumor node metastasis (TNM) classification (8).

At follow-up visits, patients underwent neck palpation and serum Tg and TgAb measurements during thyroid hormone suppression therapy, and further imaging, such as ultrasound or magnetic resonance imaging of the neck and mediastinum, when there was clinical suspicion of disease recurrence.

All patients received thyroid hormone suppression therapy at the time of sampling. Samples from small groups of patients were assayed during the initial treatment phase, after radioiodine ablation therapy or subsequent radioiodine therapy.

Tg and TgAb concentrations were measured with standard and additional methods as described below. Patients positive for TgAbs ($n = 17$), as measured in one or both quantitative TgAb assays, were excluded because of possible interference in the Tg assay. Four patients had to be excluded because of incomplete laboratory test results. Serum results from the remaining 110 patients were used for further analysis.

Disease state at the time of measurement was assessed on the basis of routine follow-up data. "No evidence of disease" was defined as absence for at least 1 year of clinically detectable disease and of Tg detection by the routinely used Tg-IRMA during thyroid hormone treatment. In case of Tg detection within 1 year after radioiodine therapy, no evidence of disease was defined as a negative diagnostic or posttherapy I-131 whole-body scan or undetectable Tg after discontinuation of thyroid hormone treatment. Patient characteristics are shown in Table 1.

Tg ASSAYS

The established ELSA-hTG (CIS Bio International) Tg-IRMA is a solid-phase 2-site IRMA that uses 2 monoclonal antibodies, one coated on a solid phase and one labeled with ^{125}I and used as a tracer. Functional sensitivity (defined as the lowest concentration with an interassay CV $\leq 20\%$), was $1.5 \mu\text{g/L}$. Interassay imprecision was 8% and 6.9% at 5 and $223 \mu\text{g/L}$, respectively. The Tg-IRMA was not calibrated against the CRM-457 reference preparation.

The Nichols Advantage[®] Tg-ICMA (Nichols Institute Diagnostics) is a fully automated 2-step chemiluminometric sandwich immunoassay that uses 3 monoclonal antibodies: 2 are biotinylated and used for capture, and the third antibody is labeled with acridinium ester for emitted-light quantification. Throughput is up to 80 samples/h with a time to first result of 51 min. The Tg-ICMA was calibrated against the CRM-457 reference preparation. The limit of detection was determined by reading the +3 SD response from 10 replicate measurements of the zero calibrator from the stored master curve on 2 different occasions. We determined functional sensitivity [defined as the lowest serum Tg concentration for which the interassay imprecision (CV) did not exceed 20%] and between-run reproducibility by measuring human DTC serum pools with Tg concentrations of 0.66, 16, and $146 \mu\text{g/L}$ in 35 runs over a 7-month period with calibration on a weekly basis using 2 different lots of reagents. We tested interference by heterophilic antibodies (HAMAs) by remeasuring Tg after incubating $500 \mu\text{L}$ of serum sample in heterophilic blocking tubes (HBTs; Scantibodies) at room temperature for 1 h.

We used a third Tg assay for method comparison in a limited number of patient sera samples. This Tg-RIA, with a functional sensitivity of $1 \mu\text{g/L}$, was reported to have minimal interference from TgAbs (4) and was developed by the University of Southern California Endocrine Services Laboratory (Los Angeles, CA).

ASSAYS FOR TgAbs

Two different quantitative assays were used for TgAb detection. The Nichols Advantage TgAb (Nichols Institute Diagnostics), with a cutoff value for TgAb positivity of 2 mIU/L, and the AxSYM TgAb assay (Abbott Laboratories), with a cutoff value for TgAb positivity of 45 mIU/L. Both TgAb assays are referenced to the WHO TgAb First International Reference Preparation (WHO 65/93).

THYROTROPIN ASSAY

Serum thyrotropin (TSH) concentrations were measured by a time-resolved immunofluorometric assay on the DELFIA system (PerkinElmer Life Sciences).

Results

ANALYTICAL PERFORMANCE OF THE Tg-ICMA

The detection limit of the Tg-ICMA was $0.05 \mu\text{g/L}$. For human DTC serum pools, the Tg-ICMA interassay imprecision over a 7-month period was 19% at $0.66 \mu\text{g/L}$, 5.5% at $16 \mu\text{g/L}$, and 12% at $146 \mu\text{g/L}$. The functional sensi-

Table 1. Clinical characteristics of studied patients.

| Clinical characteristics | All patients ^a (n = 131) | Patients included for analysis ^b (n = 110) | Patients with discordant Tg results ^c (n = 12) |
|--|--|---|---|
| M/F, n | 29/102 | 22/88 | 5/7 |
| Mean (SD) age, years | 54 (17) | 55 (17) | 45 (15) |
| Histology, n | | | |
| Papillary | 85 | 71 | 10 |
| Follicular | 39 | 32 | 1 |
| Hürthle cell | 7 | 7 | 1 |
| TNM, ^d n | | | |
| T0 | 3 | 1 | 1 |
| T1–T3 | 94 | 78 | 9 |
| T4 | 20 | 18 | 2 |
| Tx | 14 | 13 | 0 |
| N0 | 84 | 69 | 5 |
| N1 | 42 | 34 | 7 |
| Nx | 7 | 7 | |
| M0 | 118 | 97 | 12 |
| M1 | 12 | 12 | 0 |
| Mx | 1 | 1 | 0 |
| Age at initial diagnosis, ^e years | 40 (32–54) | 41 (32–54) | 37 (25–47) |
| Follow-up, ^e years | 8 (2–18) | 6 (2–19) | 6 (2–13) |
| Disease-free period, ^e years | 8 (2–18) | 9 (2–19) | 7 (3–13) |
| Disease state at time of Tg measurement, n | | | |
| No evidence of disease | 95 | 81 | 8 |
| Evidence of disease | 36 | 29 | 4 |

^a All patients enrolled at the start of the study.
^b TgAb-negative patients with complete laboratory results.
^c Patients with detectable Tg in the ICMA and undetectable Tg in the IRMA.
^d Postoperative TNM classification (8).
^e Median (interquartile range).

tivity, defined as the lowest concentration of serum Tg for which the interassay CV did not exceed 20%, was set at 0.6 µg/L, based on the CV of 19% for the mean concentration of the low serum pool (0.66 µg/L).

We used Passing and Bablok regression analysis to compare DTC sera with measurable Tg in both the Tg-ICMA and Tg-IRMA, as shown in Fig. 1. The regression analysis yielded the following equation: Tg-ICMA = 1.87 (95% confidence interval, 1.69–1.96) × Tg-IRMA + 3.01 (1.00–5.19); $r = 0.992$ (n = 40). A limited number of TgAb-negative sera (n = 8) with Tg concentrations of 2–70 µg/L according to the Tg-ICMA were also tested by Tg-RIA; Passing and Bablok regression yielded the following equation: Tg-ICMA = 1.78 (1.71–1.87) × Tg-RIA + 0.72 (0.16–1.240); $r = 0.999$. The Tg-RIA has been standardized against the CRM-457 reference preparation. A marked difference in results for the Tg-ICMA and Tg-IRMA/Tg-RIA methods was likely related to differences in monoclonal epitope specificity.

CLINICAL PERFORMANCE OF THE Tg-IRMA AND Tg-ICMA

We compared results obtained with both Tg assays and divided them into concordant and discordant results. Concordant results were Tg concentrations detectable in both assays (Tg-IRMA ≥1.5 µg/L and Tg-ICMA ≥0.6 µg/L) or

Tg concentrations undetectable in both assays (Tg-IRMA <1.5 µg/L and Tg-ICMA <0.6 µg/L). Results were considered discordant when Tg concentrations were detectable in one assay and undetectable in the other assay. Disease state and Tg results were correlated (Table 2).

CONCORDANT Tg RESULTS

Concordant results from both Tg assays were obtained for 98 patients. In 76 sera, no Tg was measurable by either the Tg-IRMA (<1.5 µg/L) or the Tg-ICMA (<0.6 µg/L), and in 22 sera Tg was measurable by both Tg assays, with Tg concentrations of 3.3–136 000 µg/L in the Tg-IRMA and 9.2 to >125 000 µg/L in the Tg-ICMA.

Clinical data showed that of 76 patients with undetectable Tg results for both assays, 69 had no evidence of disease whereas 7 had evidence of disease at the time of measurement, and all 22 patients with detectable Tg results in both Tg assays had evidence of disease at the time of measurement.

DISCORDANT Tg RESULTS

Discordant results were obtained in 12 patients (11%). In all of these patients, Tg was measurable by the Tg-ICMA (≥0.6 µg/L) but not by the Tg-IRMA (<1.5 µg/L). The median (SD) Tg results obtained with the Tg-ICMA were 1.9 (2.2) µg/L (range, 0.65–8.6 µg/L). The clinical characteristics of

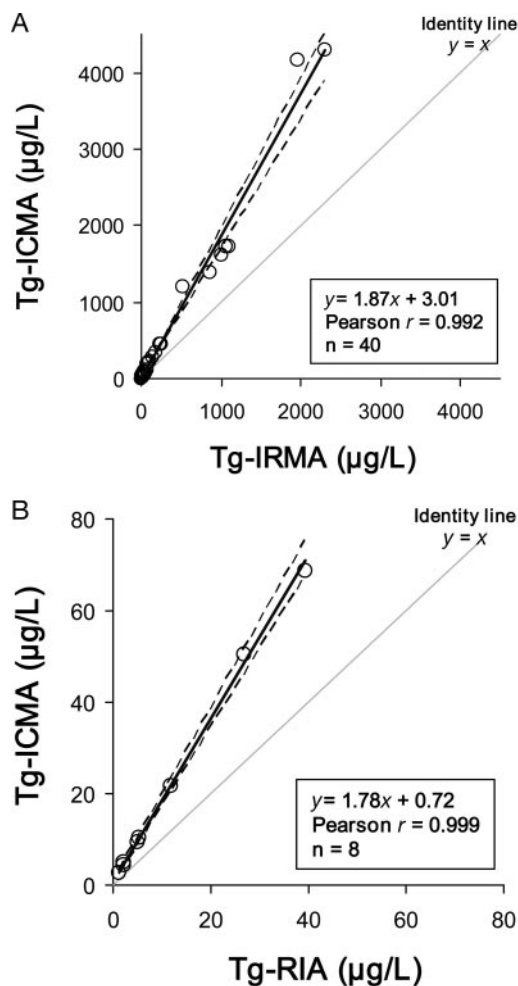


Fig. 1. Passing and Bablok regression analysis comparing the established Tg-IRMA (A) and Tg-RIA (B) with the Tg-ICMA.

Comparison of results obtained with the different Tg methods for TgAb-negative sera. A total of 40 serum samples from 22 patients with measurable Tg in both the Tg-ICMA and Tg-IRMA were used. (A), serum Tg concentrations measured by Tg-IRMA and Tg-ICMA ($n = 40$). (B), serum Tg concentrations measured by Tg-RIA and Tg-ICMA ($n = 8$). The *dashed lines* represent 95% confidence intervals. The *gray lines* indicate lines of unity.

the 12 patients with discordant Tg results are shown in Table 1. Eight of these 12 patients had no clinical evidence of disease, whereas 4 (33%), with Tg concentrations ranging from 1.5 to 4.7 $\mu\text{g/L}$, had clinical evidence of disease, based on nuclear or radiologic imaging, at the time of measurement. The clinical characteristics and disease states of these 4 patients are shown in Table 3. As shown in Table 3, 1 of these 4 patients was assessed as having "clinical evidence of disease" during the initial treatment phase.

In 1 case we observed a marked discrepancy in serum Tg concentrations measured by both methods: serum Tg was 8.6 $\mu\text{g/L}$ by the Tg-ICMA and <1.5 $\mu\text{g/L}$ by the Tg-IRMA. Because Tg was not detected (<1.0 $\mu\text{g/L}$) by the Tg-RIA, we incubated the serum in HBTs to determine whether the increased Tg result for the Tg-ICMA assay was attributable to interference from heterophilic antibodies. The Tg concen-

Table 2. Results of Tg assays divided into concordant and discordant results, related with disease state at time of measurement.

| Tg assay results | No evidence of disease (n = 77) | Evidence of disease (n = 33) | Total ^a (n = 110) |
|----------------------------------|---------------------------------|------------------------------|------------------------------|
| Concordant results | | | |
| Tg undetectable by IRMA and ICMA | 69 | 7 | 76 |
| Tg detectable by IRMA and ICMA | 0 | 22 | 22 |
| Discordant results | | | |
| Tg detectable only by ICMA | 8 | 4 | 12 |

^a TgAb-negative patients with complete laboratory test results.

tration decreased to 1.2 $\mu\text{g/L}$ after incubation in HBTs, indicating interference by heterophilic antibodies.

Discussion

In this study the analytical performance of the new ICMA was characterized by high sensitivity with a detection limit of 0.05 $\mu\text{g/L}$ and a functional sensitivity of 0.6 $\mu\text{g/L}$. Additional benefits of this ICMA were its full automation, nonradioactive design, high reproducibility, and short time to result. Clinical performance showed that for 12 of the 110 DTC patients (11%), 4 (33%) of whom had clinical evidence of disease at the time of measurement, Tg was detected by ICMA but not by IRMA.

In the postoperative follow-up of DTC patients, the main objective is the identification of patients who have residual tumor or develop a recurrence. Serum Tg detection in the follow-up phase indicates the presence of residual healthy thyroid tissue or metastatic disease (9). Thus, important clinical decisions such as whether patients should undergo diagnostic or therapeutic procedures are based on the measurement of the serum Tg concentration in individual patients (10), as is supported by the prominent place of the assay for (recombinant) TSH-stimulated Tg concentration in follow-up protocols (11, 12).

The sensitivity of Tg measurements can be optimized by clinical and technical improvements (13). Clinically, measurements of TSH-stimulated Tg after thyroid hormone withdrawal or exogenous TSH administration in patients with undetectable serum Tg during thyroid hormone suppression therapy is currently recommended for unmasking occult disease (12, 14). Technically, the development of Tg assays with improved functional sensitivity enhances the value of Tg measurements. The Tg-ICMA meets the criterion proposed by an expert panel (11, 12) that a Tg assay should have a functional sensitivity of at least 1 $\mu\text{g/L}$.

Standardization or specificity differences can lead to between-method biases. The Tg-ICMA and Tg-RIA are both standardized to CRM-457, whereas the Tg-IRMA is not. Tg-IRMA results are ~20% lower when the Tg-IRMA standardization is compared with CRM-457 (manufacturer's information). The ~2- to 3-fold higher readings obtained with the Tg-ICMA compared with both Tg-IRMA and Tg-RIA more likely reflect differences in the number and

Table 3. Clinical data of patients with detectable Tg only by ICMA assay and clinical evidence of disease.

| Patient | Age/Sex ^a | Histology | TNM ^b | Follow-up, years | Tg-ICMA, µg/L | Tg-IRMA, µg/L | Disease state ^c |
|---------|----------------------|------------------|------------------|------------------|---------------|---------------|---|
| 1. | 39/F | Pap ^d | T4N1M0 | 4 | 1.5 | <1.5 | Persistent disease; detectable Tg (IRMA) during withdrawal; negative last posttherapy scan; suspicious pre-/paratracheal neck foci on MRI |
| 2 | 55/M | Fol | T0N1M0 | 8 | 4.67 | <1.5 | Repeatedly marginally detectable Tg concentrations (IRMA) previous years; no identifiable disease remnant |
| 3 | 33/F | Pap | T2N1M0 | 0 | 2.8 | <1.5 | Initial treatment phase; abnormal neck uptake on postablation WBS |
| 4 | 41/M | Pap | T2N1M0 | 1 | 2.7 | <1.5 | Persistent disease; suspicious paratracheal mass on MRI |

^a Age in years.

^b Postoperative TNM classification (8).

^c Disease state at time of Tg measurement.

^d Pap, papillary; Fol, follicular; MRI, magnetic resonance imaging; WBS, whole-body scan.

epitope specificities of the Tg monoclonal antibody reagents used by the manufacturers and differences between the nonserum calibrator matrices and patient sera (15). Accordingly, Spencer et al. (16) recently showed wide biases among 10 tested immunometric methods and attributed this finding to differences in assay specificities for circulating Tg isoforms rather than differences in assay standardization. Despite the 2- to 3-fold higher reading obtained with the Tg-ICMA, it has a far lower detection limit (0.6 µg/L) than the Tg-IRMA (1.5 µg/L); thus, 4 additional patients with evidence of disease were identified by the Tg-ICMA but not the Tg-IRMA.

The significance of the low Tg concentrations detectable by Tg-ICMA in the remaining 8 patients in this study is unclear. Because detectable serum Tg in the follow-up phase has always been associated with the presence of residual healthy thyroid tissue or metastatic disease (9, 17), these data could identify a population at high(er) risk for recurrence. Such findings could enable risk stratification on the basis of Tg result and patient characteristics and the development of follow-up protocols more adapted to individual patients. On the other hand, lower specificity for the presence of recurrent thyroid cancer is a possible limitation of more sensitive Tg assays. Most of the 8 patients with Tg detectable by ICMA but not by IRMA and with no evidence of disease had undergone follow-up for years and had a mean disease-free period of 8 years. The present study had a cross-sectional design, and clinical evaluation was based on clinical history. Previous follow-up studies have shown that not all patients with detectable Tg will develop recurrent disease (18, 19). Zöphel et al. (20) observed that in 96% of DTC patients in remission with initial low Tg concentrations (0.03–0.8 µg/L by Tg ELISA), Tg concentrations were essentially unchanged during a 4-year observation period, and all of these group remained well. Of the 5 patients (4%) in whom Tg concentrations increased, all but 1 had recurrence of DTC.

Interpretation of the low detectable Tg values obtained in our study is uncertain, but such findings may give rise

to possibly unnecessary concern and even excessive diagnostics. Therefore, follow-up data of this Tg assay are required to interpret the finding of isolated Tg values and justify the performance of additional diagnostics in this group of patients. Moreover, because a change in serial Tg measurements during follow-up may be more informative for recurrence of disease than an absolute value of Tg in the lower range, prospective data for these patients could provide valuable information (20).

Although sensitivity is optimized in this assay, 7 patients with evidence of disease had Tg concentrations below the functional sensitivity of the ICMA, suggesting that the sensitivity is suboptimal for managing patients with DTC (21). Moreover, Spencer et al. (16) recently showed that interference in Tg measurement by TgAbs cannot be excluded when TgAbs are not detectable. Tg was reported undetectable in euthyroid controls without evidence of TgAbs. Consequently, “confidence in the specificity of a negative antibody report with an undetectable serum Tg becomes less secure” (21). Furthermore, TgAb assays vary considerably in sensitivity and specificity, probably because of differences in assay specificities for the conformational epitopes characteristic of endogenous TgAbs (16). Therefore, in our study, the 7 patients with evidence of disease but undetectable Tg by ICMA may have serum TgAbs that cannot be detected by the methods used. Similarly, Spencer et al. (16) reported poor concordance of TgAb detection among 12 direct TgAb methods. In addition, tumor dedifferentiation can lead to an absence of Tg synthesis or synthesis of Tg with altered biochemical features, obscuring recognition by the antibodies used in the Tg assay (22). Therefore, low or even undetectable Tg does not guarantee absence of recurrent or metastatic disease (22, 23).

Immunometric methods can also be subject to interference from HAMAs, leading to inappropriately high serum Tg values (5, 6). Recently, Preissner et al. (5) showed that HAMA interference is relatively prevalent (1.5%–3%) in a commonly used automated Tg assay and can lead to clinically significant artifacts. The Tg-ICMA appears to

suffer from similar problems, as shown in the case reported here, despite the fact that the manufacturer has added mouse serum in the assay procedure as a precautionary measure. The possibility that HAMA interference played a role in the other discrepant cases in our study cannot be ruled out but is unlikely because the discrepancies in these cases can be explained by the difference in absolute readings between the Tg assays. However, interference from HAMAs should be considered if the Tg result does not fit the clinical picture. Further investigation, by repeated testing with a different Tg assay, testing serial dilutions, or treatment with additional blocking reagents, is advocated.

In conclusion, the new ICMA is a robust and sensitive Tg assay that optimizes the identification of patients with disease activity during follow-up of DTC. Because Tg is detectable by the ICMA in some patients with no clinical evidence of disease, follow-up data on these patients are needed to demonstrate the applicability of this Tg assay in clinical practice.

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