Evaluation of Highly Sensitive Thyrotropin Assay for Detecting Thyroid Diseases in Neonatal Screening: Preliminary Studies

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Abstract. TSH concentrations in dried blood samples on filter paper were determined by a conventional enzyme-linked immunosorbent assay (ELISA), used for routine neonatal screening for primary hypothyroidism, and a highly sensitive bioluminescence ELISA (BL-ELISA) using firefly luciferase to examine whether central hypothyroidism and hyperthyroidism can be efficiently detected as cases of primary hypothyroidism. Samples were obtained from 3 patients with congenital central hypothyroidism, 5 patients with congenital primary hypothyroidism, 6 patients with hyperthyroidism, 31 neonatal babies with low birth weight (premature babies) and 242 newborn babies with normal birth weight from the general population (normal babies). The TSH values were low in central hypothyroidism and hyperthyroidism. Their deviations from the mean TSH value for normal babies by the BL-ELISA method (-3.12 SD and -4.79 SD in central hypothyroidism and hyperthyroidism respectively) were greater than those by the ELISA method (-2.00 SD and -2.97 SD respectively). The TSH values were high in primary hypothyroidism and normal in premature babies while deviations were the same when BL-ELISA and ELISA were used. These findings indicate that the highly sensitive TSH assay (BL-ELISA) can be used for detecting both primary and central hypothyroidism as well as hyperthyroidism in neonatal screening.

Key words: Neonatal screening, Central hypothyroidism, Sensitive TSH assay, Firefly luciferase
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SINCE irreversible mental retardation in neonatal hypothyroidism can be prevented by early diagnosis and treatment, neonatal mass screening has been widely used [1]. At present, measure-

ments of thyroxine (T_4) or TSH in dried blood samples on filter paper taken from neonates are used for neonatal screening worldwide. The total T_4 method [2] can detect cases of primary and central hypothyroidism with decreased T_4 , but gives false-positive results for subjects with T_4 binding globulin (TBG) deficiency who do not need treatment. The free T_4 method [3–5] can also detect primary and central hypothyroidism and is not influenced by TBG deficiency, but both methods

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of measuring total and free T₄ risk overlooking cases of mild hypothyroidism (false-negative) and give false-positive results for premature babies with transient, subnormal total or free T₄. On the other hand, the TSH method [6–9] is useful in detecting even mild cases of primary hypothyroidism with increased TSH, without giving false-positive results for subjects with TBG deficiency and premature babies. But this method can not detect central hypothyroidism, since the sensitivity of the conventional TSH method (ELISA) is too low to detect decreased TSH.

Recently, a highly sensitive bioluminescence ELISA (BL-ELISA) with biotinylated firefly luciferase [10] as a marker enzyme has developed and this method may detect decreases in TSH. We therefore used this method to evaluate the efficiency in detecting central hypothyroidism and hyperthyroidism with decreased TSH as well as primary hypothyroidism in neonatal screening.

Subjects and Methods

Subjects

Dried blood samples were obtained on filter paper from 287 subjects at neonatal screening (between 3 and 5 days of age) or in adults.

Three patients with congenital central hypothyroidism, (cases 1–3) were studied. Cases 1 and 2 were sister offspring of a consanguineous marriage. They were born at full term with normal birth weight in 1954 and 1956 when the neonatal screening program had not yet been conducted. They had typical signs and symptoms of congenital hypothyroidism. The clinical diagnosis of isolated TSH deficiency was confirmed at ages of 14 and 12 years by laboratory findings: low serum free T₄ index, undetectable TSH, no response of TSH to TRH administration and normal secretion of other pituitary hormones [11]. A missense mutation in the TSH β -subunit gene was demonstrated 20 years later [12]. Dried blood samples were obtained from the patients at ages of 32 and 30 years under the hypothyroid condition after discontinuation of medication with thyroid hormone (serum free T₄ was less than 0.1 ng/dl and TSH was less than 0.1 mU/l). Case 3 was born at full term to non-consanguineous healthy parents [13]. She weighted

3662 g at delivery but her postnatal growth was severely retarded. The diagnosis of combined deficiencies of TSH, PRL and GH caused by a missense mutation in the transcription factor PIT1 gene was confirmed between 5 and 8 months of age. Laboratory findings showed that serum TSH was not high (1.2 and 1.5 mU/l) and showed a little response to TRH administration (the peak TSH value was 2.5 mU/l) but T₄ (0.7 and 2.8 μ g/dl) and free T₄ (0.7 and 0.7 ng/dl) were low, and this was consistent with the diagnosis of central hypothyroidism. A dried blood sample obtained from the patient at the neonatal screening (the 5th day) was used for the present study.

Five patients with congenital primary hypothyroidism (cases 4–8) were detected from among the general population by the neonatal screening and the confirmatory diagnosis was made by low serum T₄ or free T₄ and high TSH (492, over 200, 51, 367 and 34 mU/l respectively). Dried blood samples obtained at the neonatal screening were used.

Six patients with hyperthyroidism (cases 9–14) were studied. Case 9 was born at 34 weeks and 5 days of gestational age. He weighed 1752 g and had respiratory distress. At 19 days of age, the clinical diagnosis of neonatal hyperthyroidism was confirmed by the laboratory findings of low serum TSH (less than 0.1 mU/l), high free T₄ (over 8.4 ng/dl) and free T₃ (over 2.5 pg/dl). His thyroid function tests were normalized by treatment with an antithyroid drug for 20 days. His mother had been suffering from autoimmune thyroid disease. A dried blood sample obtained from the patient at 19 days of age was used. Cases 10–14 were adult patients with overt hyperthyroidism due to Graves' disease. Dried blood samples were obtained from the patients when clinical diagnosis was confirmed by high serum free T₄ and/or free T₃ and undetectable TSH (less than 0.1 mU/l).

Dried blood samples were obtained from 273 newborn babies from the general population at the neonatal screening. They were 31 premature babies with a low birth weight of less than 2500 g (cases 15–45) (gestational age 34.8 ± 3.4 weeks (mean and standard deviation), birth weight 1886 ± 452 g) and 242 normal babies with normal birth weight (cases 46–287) (gestational age 40.0 ± 1.4 weeks, birth weight 3076 ± 348 g).

Methods

The modified ELISA used was essentially the same as previously described [9]. Conventional ELISAs were performed with a commercial kit (CTSH II ELISA® from Eiken Chemical Co., Ltd., Japan). Briefly, each 3 mm diameter blood disc punched out from dried blood samples on filter paper, and 100 μ l of horseradish peroxidase-anti-TSH mouse monoclonal antibody conjugate in buffer (tricine, containing Triton, fetal calf serum (FCS), bovine serum-albumin (BSA), and -gamma globulin, pH 8.5) were each placed in the wells of a microplate with 96 wells altogether (NUNC Maxsorp, Denmark) which were coated with an immunoglobulin G-fraction of anti-TSH mouse monoclonal antibody. After incubation at 2–8 °C for 16–24 h, each well was washed four times with 300 μ l of washing solution (phosphate buffer containing NaCl, Tween 20). Then 100 μ l of substrate (o-phenylenediamine) was added and incubated at 25 °C for 30 min. After 100 μ l of stop solution (3N H₂SO₄) was added, absorbency at 490 nm was measured automatically.

The BL-ELISAs were carried out as follows. Each 3 mm diameter blood disc and 100 μ l of biotin-anti-TSH monoclonal antibody conjugate in buffer (Tris containing FCS, EDTA, pH 8.0) were placed in an anti-TSH coated well of a microplate (Dynex Technologies, Inc). After incubation at 2–8 °C for 16–24 h, each well was washed four times with washing solution. Then 100 μ l of biotinylated firefly luciferase (bL248)-avidine complex [10] in buffer (phosphate buffer containing BSA, casein, EDTA and 2-mercaptoethanol, pH 7.5) was added and incubated at 25 °C for 15 min. After each well was washed as described above, 100 μ l of substrate (luciferin in tricine containing ATP, EDTA, pH 7.65) was added serially and bioluminescence intensity was determined automatically with a luminometer (Labsystem Luminuskan® USA). Minimal detectable doses which were estimated from the point where the upper 95% confidence limits of the response at zero intersect the standard curve were 0.20 mU/l (ELISA) and 0.04 mU/l (BL-ELISA), respectively.

Statistical analysis

Since the distribution on TSH values in normal babies was assessed as a logarithmic-normal distribution by analysis with log-normal paper, all TSH values were transformed to logarithmic values ($X = \log_e$ TSH value). The deviation (d) of the X value in each patient from the mean found in normal babies was calculated parametrically as follows: $d = (X - M) / SD$, where M is the mean, and SD is the standard deviation of X values in normal babies. The differences between the two groups were analyzed parametrically by t -test. On the other hand, X values in normal babies were ranked, and percentiles from the lowest (0) to the highest (100) value were calculated.

Results

The data are summarized in Table 1. The TSH values and their logarithmic values (X) in congenital central hypothyroidism and hyperthyroidism were lower than those in normal babies. The deviations of X values in these patients from the mean value for normal babies determined by BL-ELISA (d_B) were significantly greater than those by ELISA (d_E). ($d_B = -3.12 \pm 0.55$ (mean and standard deviation) *vs.* $d_E = -2.00 \pm 0.37$ ($P < 0.05$) in central hypothyroidism and $d_B = -4.79 \pm 1.04$ *vs.* $d_E = -2.97 \pm 0.18$ ($P < 0.002$) in hyperthyroidism). The percentiles of TSH values in these patients were also low. In primary hypothyroidism, TSH values were significantly higher than those in normal babies but the deviations and percentiles were the same when the BL-ELISAs and ELISAs were used. There were no significant differences in the TSH values for the premature and normal babies.

Discussion

Since TSH in blood is mainly distributed in plasma and not in blood cells, TSH values determined in dried blood samples on filter paper (TSH in whole blood) must be lower than those in serum when an identical sample is used. In the present experiment, however, TSH values in dried blood samples were sometimes higher than those

Table 1. TSH concentrations determined by ELISA and BL-ELISA in dried blood samples obtained from thyroid diseases, premature babies and normal babies

Diagnosis	Case No.	Dried blood samples on filter paper							
		E L I S A				BL-ELISA			
		TSH (mU/l)	log* ¹ (X _E)	dev* ² (d _E)	per [rank]* ³	TSH (mU/l)	log* ¹ (X _B)	dev* ² (d _B)	per [rank]* ³
Congenital central hypothyroidism	1	0.60	-0.51	-1.58	5.76[14]	0.16	-1.83	-3.52	0.41[1]
	2	0.39	-0.94	-2.22	1.23[3]	0.18	-1.71	-3.35	0.41[1]
	3	0.39	-0.94	-2.22	1.23[3]	0.32	-1.14	-2.49	0.82[2]
	M	0.46	-0.80	-2.00	2.74	0.22	-1.55	-3.12	0.55
	SD			0.37				0.55	
						P<0.05* ⁴			
Congenital primary hypothyroidism	4	>100	> 4.61	> 6.06	100[243]	> 100	> 4.61	> 6.06	100[243]
	5	>100	> 4.61	> 6.06	100[243]	> 100	> 4.61	> 6.06	100[243]
	6	45.4	3.82	4.91	100[243]	48.4	3.88	5.00	100[243]
	7	>100	> 4.61	> 6.06	100[243]	> 100	> 4.61	> 6.06	100[243]
	8	38.6	3.65	4.66	100[243]	41.6	3.73	4.77	100[243]
Hyperthyroidism	9* ⁵	0.20	-1.56	-3.14	0.41[1]	0.04	-3.21	-5.71	0.41[1]
	10	0.22	-1.51	-3.07	0.41[1]	0.13	-2.04	-3.83	0.41[1]
	11	0.29	-1.24	-2.66	0.41[1]	0.10	-2.30	-4.23	0.41[1]
	12	0.21	-1.56	-3.14	0.41[1]	0.16	-1.83	-3.52	0.41[1]
	13	0.25	-1.39	-2.89	0.41[1]	0.04	-3.21	-5.71	0.41[1]
	14	0.25	-1.39	-2.89	0.41[1]	0.04	-3.21	-5.71	0.41[1]
	M	0.24	-1.44	-2.97	0.41	0.09	-2.63	-4.79	0.41
	SD			0.18				1.04	
						P<0.002* ⁴			
Premature babies (31 cases)	15~45								
	M	2.59	0.62			2.57	0.63		
Normal babies (242 cases)	46-287								
	M	2.18	0.55(M _E)			2.20	0.57(M _B)		
		SD	0.67(SD _E)				0.66(SD _B)		

>: higher, <: lower. M, mean; SD, standard deviation. *¹ log: X_E or X_B = log_e (TSH value). *² dev: d_E = (X_E - M_E)/SD_E, d_B = (X_B - M_B)/SD_B. *³ per[rank]: percentile and [ranking] from the lowest TSH value for normal babies. lowest: 0[1], highest: 100[243] *⁴ t-test, n.s.: not significant. *⁵ neonatal hyperthyroidism.

in serum, particularly when TSH in samples from subjects with hyperthyroidism and central hypothyroidism were determined by the conventional ELISA method. The reason for the discrepancy is unclear but some matrix effects are probably involved and the between-methods' variation cannot be avoided. We therefore used deviations of log (TSH) values for each subject from the mean found in normal babies instead of absolute TSH values to assess the two methods in differentiating abnormal from normal subjects.

In the present study the highly sensitive BL-ELISA with biotinylated firefly luciferase was found to be more effective than the conventional ELISA method in differentiating the low TSH values of

central hypothyroidism from those of normal babies in the general population. Since the deviations in the TSH values for the patients were so large (-3.12 SD and 0.55 percentile), the cut-off point may easily be determined. The incidence of congenital central hypothyroidism has been found to be as low as about 1:80,000 in the general population [14]. Since, however, case reports have been increasing and the incidence is high in one restricted area, the western part of the Shikoku island in Japan, mass screening may be useful in such areas [15].

While it may give false-positive results in screening for hypothyroidism, it may be useful in detecting neonatal hyperthyroidism. Mortality for

this disorder has been estimated to be as high as 25% in the past, although estimates are considerably lower now [16]. When mothers are treated with antithyroid drugs, patients with neonatal hyperthyroidism do not always show overt signs and symptoms during screening. Therefore the effectiveness of mass screening for early diagnosis of this disease is uncertain, and will remain so unless a pilot study on the general population is done.

Like the conventional ELISA of TSH, the sensitive BL-ELISA is not expensive and efficiently detects primary hypothyroidism without giving false-positive results for premature babies. Further studies are required to evaluate the total cost-benefit ratio of neonatal mass screening for detecting congenital central hypothyroidism and neonatal

hyperthyroidism by means of a single assay of TSH for primary hypothyroidism with the highly sensitive BL-ELISA.

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