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Original Article

Results from 28 Years of Newborn Screening for Congenital Adrenal Hyperplasia in Sapporo

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Abstract. The primary goal of newborn mass screening (MS) for congenital adrenal hyperplasia (CAH) is the prevention of life-threatening salt-wasting crisis in the most severe forms of CAH, and MS for CAH has been implemented in several countries. We summarize here our experience and results from newborn CAH MS from 1982 to 2010 in Sapporo City. During these 28 yr, the level of 17-hydroxyprogesterone (17-OHP) was determined in MS of samples from 498,147 newborns. During this period, 26 individuals (19 females and 7 males) with 21-hydroxylase deficiency (21-OHD) were detected. Of the 26 CAH, 20 were classified as having the salt-wasting (SW) form, 4 were classified as having the simple virilizing (SV) form, and 2 were classified as having the noncalssic (NC) form. Therefore, the frequency of the classical type of CAH was 1 in 20,756. In order to improve the effectiveness, we employed high-performance liquid chromatography (HPLC) as a second tier test from 2000. During this period, among the recalled babies, 75.4% were born prior to the 37th wk of gestation age, and the recall rate was 5.38% for premature neonates and 0.06% for mature neonates. MS for CAH in Sapporo is effective for the identification of the SW and SV forms of 21-OHD. However, the recall rate of premature babies is still high after the introduction of HPLC as a second tier test.

Key words: newborn mass screening, congenital adrenal hyperplasia, 17-hydroxyprogesterone, 21-hydroxylase deficiency, recall rate

Introduction

Congenital adrenal hyperplasia (CAH) is characterized by impaired biosynthesis of cortisol and aldosterone and increased secretion of 17-hydroxyprogesterone (17OHP) and androgens (1, 2). The most common form is 21-hydroxylase deficiency (21-OHD) (1, 2), which affects about 1 child in 18, 000 and results in symptoms that vary with the severity of the enzymatic defect. Classic forms include the salt-wasting (SW) form,

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for which there is a high risk of life-threatening adrenal insufficiency during the first month of life, and the simple virilizing (SV) form. In both cases, female neonates present with markedly virilized external genitalia. Nonclassic (NC) form can manifest with hyperandrogenism later in life and do not warrant early detection through neonatal screening (3, 4). The severity of the clinical presentation depends on the degree of 21-OHD, which is usually caused by deletion or mutations of *CYP21A2* (1, 2).

The primary goals of newborn mass screening (MS) for CAH are the prevention of life-threatening salt-wasting crisis in the most severe forms of CAH and the prevention of permanent negative effects of androgen overproduction in the SV form (2, 4, 5). MS for CAH from dried blood spots (DBS) has been implemented in several countries (3–11). An increased 17-OHP concentration in heel-prick blood is used to indicate patients at risk of having CAH. However, compared with the detection of other diseases by MS, CAH screening is associated with a high frequency for the requirement of a second sample (recall), because preterm infants have elevated 17-OHP levels due to several stresses (5, 7, 11–13). In order to increase the efficiency of CAH MS, body weight- or gestational age-adjusted cutoff levels for 17-OHP have been used (11–13). However, the false positive rate (FPR) has not been significantly improved by these methods (14).

Most recently, Sarafoglou *et al.* (14) highlighted the methodology of comparing the first tier with repeat screening and second tier screening using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for CAH.

While Japan initiated national newborn MS for CAH in 1989 (4, 15), a pilot newborn MS for CAH study was started in Sapporo in 1982 after the assessment of its feasibility and efficiency (16, 17). The aim of our study was to summarize the results of the past 28 yr of newborn MS for CAH in Sapporo and analyze the efficiency of high-performance liquid chromatography (HPLC) as a second tier test.

Methods

Screening population

From 1982 through March 2010, the entire neonatal population of 498,147 newborns was screened for CAH in Sapporo. Blood was usually collected from 4- to 6-d old infants on filter paper and sent by mail to the Sapporo City Institute of Health. Blood samples of neonates suspected to have 21-OHD were collected from day 0 to 3 d after birth.

Abnormal screening results for CAH were directly reported by Sapporo City Institute of Health to coordinating physicians at Hokkaido University Hospital or NTT East Japan Hospital. The coordinating physician traced the individual in question and decided whether the child should have a second heel puncture or should be referred to a pediatric endocrinologist immediately.

Measurement of 17-OHP from DBS and cut-off levels

The methods of measurement of 17-OHP from DBS are summarized in Table 1. Both the method of the determination of 17-OHP and cutoff levels were altered at different points during the screening period (16, 17). As a first tier test, from 1982 to 1985, 17-OHP levels were measured by an in-house ELISA kit after extraction by diethyl ether. From 1986 onwards, 17-OHP levels were measured using a commercially available ELISA kit (Enzaplate-N-17α-OHP® from 1986 to 2001, Enzaplate-Neo-17α-OHP® from 2002 to 2010, Siemens Japan, Tokyo, Japan) without prior extraction. Cutoff levels for 17-OHP in the first and second tests are also summarized in Table 1. From 1986 to 1999, as a 2nd tier test and recall test, we determined 17-OHP/cortisol directly from DBS (18). From 2000 onward, as a second tier test, we employed HPLC to determine 17-OHP. The method of HPLC was reported previously (19). In addition, cutoff levels for 17-OHP in recall DBS are summarized in Table 1. From 2000, 17-OHP in recall DBS was also measured by HPLC.

Table 1 Methods and cutoff levels of CAH screening

Year	Reagent	1st DBS				Recalled DBS	
		1st tier test		2nd tier test		Method	Cutoff value for medical evaluation
		Method for 17-OHP	Cutoff value for 2nd tier test*	Method for 17-OHP	Cutoff value for recall		
1982	Made in-house	ELISA-extract †	>97 percentile	ELISA-extract	Mean+3SD	ELISA-extract	>5 ng/ml
1983–1985	Enzaplate N-17αOHP	ELISA-extract	>97 percentile	ELISA-extract	>5 ng/ml	ELISA-extract	>5 ng/ml
1986–1999	Enzaplate N-17αOHP	ELISA-direct ‡	>97 percentile	ELISA-extract	>7 ng/ml	ELISA-extract	>7 ng/ml
				Cortisol	17-OHP/cortisol >0.8§	Cortisol	17-OHP/cortisol >0.8
2000–2001	Enzaplate N-17αOHP	ELISA-direct ‡	>97 percentile	ELISA-extract	>7 ng/ml	ELISA-extract	>7 ng/ml
				HPLC	>2.5 ng/ml	HPLC	>2.5 ng/ml
2002–2005	Enzaplate Neo-17αOHP	ELISA-direct ‡	>97 percentile	ELISA-extract	>4.5 ng/ml	ELISA-extract	>4.5 ng/ml
				HPLC	>2.5 ng/ml	HPLC	>2.5 ng/ml
2006–2010	Enzaplate Neo-17αOHP	ELISA-direct ‡	>5.5 ng/ml	HPLC	>2.5 ng/ml	HPLC	>2.5 ng/ml

* First tier sample automatically sent for second tier test. † ELISA-extract, the assay after extraction of 17-OHP by diethyl ether. ‡ ELISA-direct, 17-OHP was measured without extraction by diethyl ether. § The ratio of directly measured 17-OHP/cortisol in DBS.

Table 2 Numbers of recalled babies, recall rates and numbers of medical evaluations from 1995 to 2010

Year	Gestational weeks	No. of screened neonates	No. of recall	Recall rates (%)	No. of medical evaluation after recall (%)
From 1995–1999	Total	87, 194	211	0.24	7 (0.008)
	<37 wk	4,929	128	2.60	3 (0.06)
	≥37 wk	82,114	81	0.10	4 (0.005)
From 2000–2010	Total	164,798	669	0.41	6 (0.004)
	<37 wk	10,780	580	5.38	2 (0.02)
	≥37 wk	153,966	89	0.06	4 (0.003)

Clinical and genetic analysis

Where available, clinical data were collected, including birth weight, gestational age, sex, 17-OHP levels at first screening, day of referral to the hospital, day at start of treatment and serum Na^+ and K^+ levels at first evaluation. The genotype of *CYP21A2* was determined as previously reported (20, 21).

Patients were defined as having the SW form when they had a serum sodium concentration <135 mEq/l or required fludrocortisone and NaCl supplementation for normal weight gain during hydrocortisone therapy. Patients were defined as having the SV form when they were treated with hydrocortisone only and had no symptoms of salt wasting. Patients were defined as having the NC form when they showed no symptoms.

Results

Results of screening

During the 28-year study period, a total of 498,147 newborns were screened for CAH. Of the 26 CAH patients, 20 were classified as having the SW form, 4 were classified as having the SV form, and 2 were classified as having the NC form. Therefore, the frequency of the classical type of CAH was 1 in 20,756.

Of these, 26 individuals (weight range 2,340–3,774 g) were diagnosed with 21-OHD. In order to decrease the recall rate, we tried different methods for the second tier test, as shown in Table 1. While we did not employ birth weight and/or gestationally-adjusted cutoff levels for 17-OHP, the gestational week of neonates has been registered since 1995.

The recall rates are summarized in Table 2. From 1995 to 1999, 87,194 screening tests for CAH were performed. In this period, the total recall rate was 0.24%. The recall rates for premature and mature babies were 2.60 and 0.10%, respectively. From 2000 to 2010, 164,798 screening tests for CAH were performed, and 669 neonates required a second DBS after a second tier test, corresponding to a total recall rate of 0.41%. The recall rates for premature and mature babies were 5.38 and 0.06%, respectively.

The numbers of medical evaluations after recall are also summarized in Table 2. From 1995 to 1999, seven babies (0.008%) were subjected to medical evaluation in hospitals were after recall, and none of them were diagnosed as having 21-OHD. From 2000 to 2010, six babies (0.004%) were subjected to medical evaluation in hospitals after recall, and two of full-term infants were diagnosed as having 21-OHD (patient 17 and 24 in Table 3).

Characteristics of 21-OHD patients detected by MS

The characteristics of 21-OHD patients detected by MS are summarized in Table 3. Of 26 CAH patients, 20 were classified as having the SW form, 4 were classified as having the SV form, and 2 were classified as having the NC form. There were 19 female patients and 7 male patients. Two familial cases (patients 1 and 3 and patients 9 and 13) were detected. The mean day at examination was 6.4 d after birth (range=0 to 18 d). Among the 18 female patients, 11 patients were suspected as having CAH prior to the MS result becoming available as a result of detection of virilization and were referred to neonatal centers. Five female patients were referred after MS screening.

One patient (patient 17) had normal female genitalia and no symptoms; however, repeated measurements detected elevated levels of 17-OHP, and an ACTH stimulation test was performed at 2 mo of age. After ACTH stimulation, the serum 17-OHP level rose from 2.6 to 86.6 ng/ml. Based on these results, she was diagnosed as having the NC form (2). She is currently 11 yr old and shows no symptoms of androgen excess; however, her serum 17-OHP level remained high (4–9 ng/ml). In contrast to the female patients, all male CAH patients were diagnosed by MS. In the 21-OHD patients for whom we had data, the average day of initiation of therapy in those patients who had

Table 3 Characterization of patients with 21-OHD diagnosed from 1982 to 2010

Patient	Sex	Birth weight (g)	GA	17-OHP (ng/ml) at the first screening	Method of measurement of 17-OHP	Day at screening	Day at examination	Serum Na (mEq/L)	Serum K	Day at start of treatment	Genotype of *CYP21A2*	Type of disease
1	F	2,720	NA	>1,000	Made in-house	2	10	139	6.3	NA*	Not determined	SW
2	F	3,410	41W	109	Made in-house	5	13	137	4.5	NA	IVS2-13A/C>G/I172N	SV
3	F	2,750	39W	507	Made in-house	2	3	137	4.5	NA	Not determined	SW
4	M	2,829	36W	465	Made in-house	5	13	134	5.4	NA	Del§/IVS2-13A/C>G	SW
5	F	3,140	38W	225.3	N-17αOHP†	1	4	138	4.2	NA	Not determined	SW
6	M	3,690	40W	69.8	N-17αOHP	5	NA	NA	NA	NA	IVS2-13A/C>G/I172N	SV
7	F	2,640	38W	>300	N-17αOHP	4	1	140	5.9	5	Del/Q318X+R356W	SW
8	F	3,780	41W	136	N-17αOHP	2	3	140	6.2	4	Del/IVS2-13A/C>G	SW
9	F	2,870	37W	489	N-17αOHP	7	11	NA	NA	11	Del/IVS2-13A/C>G/	SW
10	F	3,190	41W	243	N-17αOHP	1	1	137	6.2	7	Del/Q318X+R356W	SW
11	F	2,698	35W	>81	N-17αOHP	7	12	128	7.3	12	Del/not found	SW
12	M	3,364	40W	312.2	N-17αOHP	5	11	129	7.8	11	Del/R356W	SW
13	F	2,890	41W	550.2	N-17αOHP	7	0	143	4.8	6	De//IVS2-13A/C>G	SW
14	M	3,692	39W	178.7	N-17αOHP	7	11	118	7.8	11	Del/Q318X+R356W	SW
15	F	3,995	40W	43	N-17αOHP	5	18	133	5.7	21	I172N/Del	SW
16	F	2,664	38W	324.1	N-17αOHP	2	0	NA	NA	NA	Del/not found	SW
17	F	3,618	39W	41.9	N-17αOHP	5	14	141	5.2	No treatment	Not found	NC
18	F	3,048	38W	281.4	Neo-17αOHP‡	5	10	139	7.1	10	Del or Conv§§/I172N	SW
19	F	3,000	40W	471.3	Neo-17αOHP	0	0	141	4.5	3	Not determined	SV
20	M	2,304	36W	>100	Neo-17αOHP	5	7	130	6.4	7	Not determined	SW
21	F	3,082	40W	>100	Neo-17αOHP	1	1	142	4.9	2	Del/IVS2-13A/C>G	SW
22	M	3,212	40W	>100	Neo-17αOHP	5	12	122	8.2	14	Not determined	SW
23	F	2,650	39W	>100	Neo-17αOHP	3	1	145	4.9	6	Del/IVS2-13A/C>G/	SV
24	M	2,770	37W	10.2	Neo-17αOHP	5	14	142	4.0	21	P30L/I172N+R356W	NC
25	F	3,774	40W	>100	Neo-17αOHP	1	1	137	5.5	2	Not determined	SW

* NA: not available. † Enzaplate N-17αOHP, ‡ Enzaplate Neo-17αOHP, §Del, deletion of *CYP21A2*. §§Conv, partial conversion of *CYP21A2*.

undergone glucocorticoid therapy was 8.2 d after birth (range=2 to 21 d). Failure to thrive, severe hyponatremia, and hyperkalemia requiring immediate intravenous fluid replacement therapy were observed in two male patients (patient 14 and 22). In 4 patients (2 girls and 2 boys), laboratory signs of beginning salt wasting were found at the time of medical examination (sodium, <135 mEq/l). Of the remaining patients, 13 showed no detectable signs of salt wasting at the first examination; however, during follow-up, 11 patients demonstrated reduced serum sodium levels and poor weight gain despite appropriate glucocorticoid therapy. Accordingly, supplemental fludrocortisone and NaCl were administered in addition to hydrocortisone, and these patients were classified as having the SW form.

In patient 22, the time of referral to our hospital was 12 d after birth. Due to the time taken to contact the parents of the patient, performance of a full medical examination was delayed.

Regarding the genotype of *CYP21A2,* the most frequent genetic defects were deletions of *CYP21A2* and IVS2-13A/C->G, which is in agreement with other studies (1, 2). Two SV patients had an I172N mutation in at least one allele. This mutation resulted in an enzyme with a small amount of activity. We were unable to determine the genotype of the NC patient (patient 17) using the conventional methods we employed. Patient 24 (NC) showed a very slight elevation of 17-OHP in the MS, which was verified by repeated measurement. As he did not have any symptoms, he was considered to have the NC form of 21-hydroxylase deficiency. Genetic analysis of *CYP21A2* showed that this patient was a compound heterozygote for P30L and I172N+R356W. P30L has been reported as a very mild mutation (1, 2), and this genotype could explain his mild phenotype.

Discussion

We summarized the results of neonatal MS for CAH in Sapporo. MS detected 26 patients affected by 21-OHD, and no other type of CAH was detected. Among them, 24 patients were diagnosed with the classical type of CAH. Therefore, the frequency of the classical type of CAH was 1 in 20,756, which is almost identical to the worldwide incidence of CAH (4–9). On the other hand, the incidence of 21-OHD in Japan during the prescreening era was reported as 1 in 43,764 (15). These findings confirm that some 21-OHD patients went undiagnosed during the prescreening era and that screening for CAH has improved the rate of case detection by one-third.

The sex distribution of CAH patients in our study was 19 girls (73%) and 7 boys (27%). While a lower prevalence of MS-detected CAH in males has been reported in another study in Europe (22), our number of patients is currently of insufficient size to discuss sex distribution.

All male patients were detected by screening, as were 5 of 18 female neonates. Eleven of 18 female newborns were identified by genital abnormalities before screening, indicating that aside from the NC cases, 50% of the 21-OHD patients in our study were identified by MS. Other studies have shown that clinical criteria alone are insufficient to detect about 50% of CAH cases in the newborn period (22), a rate comparable with our results.

In our study, the average day of initiation of therapy was 8.2 d after birth (range=2 to 21 d). This is later than the 6.7 d reported by Steigert *et al.* (10) but earlier than the 17.6 d reported by Suwa *et al.* in a previous study in Japan (15).

Our screening identified two patients (patients 17 and 24) with the NC form, even though our screening program was not specifically designed to detect this form of CAH. It has been reported that several cases of the NC form showed elevated 17-OHP levels and were identified by neonatal screening (2, 3, 23, 24). Identification of this form would require a lower

cutoff value and entail a significant increase in false positive recalls. Therefore, some cases with the NC form are thought to have been missed by our screening.

MSs for CAH are still suboptimal because of low specificity, particularly in premature infants. To reduce the recall rate, we tried several methods of second tier test. In addition to the measurement of 17-OHP in DBS with extraction, from 1986 to 1999, the ratio of 17-OHP/cortisol was used, and from 2000 to 2010, HPLC was used. As a result, the total recall rate increased from 0.24 to 0.41% after the introduction of HPLC. The recall rate increased particularly in preterm infants, from 2.60 to 5.38%. One of the reasons for this might be our adoption of a low cutoff value of 17-OHP as a second tier test (2.5 ng/ml). A low cutoff value increases the recall rate, while a higher cutoff value would result in a loss of sensitivity. It must be kept in mind that the appropriate cutoff level of 17-OHP is still debatable.

If only term neonates are considered, HPLC decreased the recall rate (from 0.10 to 0.06%). Furthermore, the rate of medical evaluation after recall decreased (from 0.008 to 0.004%). Thus, HPLC is considered to be effective for these two points in term neonates. However, HPLC is time-consuming (one analysis takes 40 to 50 min), and the difficulty of analyzing many samples using this method has limited its adoption in Japan. To avoid unnecessary recalls, several studies in the USA and Europe have reported the development of a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay as the second tier test for CAH screening (14, 25, 26). This method can detect not only levels of 17-OHP but also those of other steroids such as androstenedion and cortisol, resulting in a decrease in recalls for CAH MS. In Japan, LC-MS/MS has been employed for neonatal screening of congenital metabolic diseases (27), and measurement of several steroids by LC-MS/MS has been reported (28). A pilot study of LC-MS/MS as the second tier test for CAH screening is now ongoing (29). In the future, LC-MS/MS will be used as the second tier test for CAH, and we anticipate a further increase in screening efficiency.

In conclusion, MS for CAH in Sapporo is useful for identification of the SW and SV forms of 21-OHD. However, more efficient methods of MS for CAH should be developed in the future.

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