Endocrine Journal 2013, **60** (7), 903-912

ORIGINAL

Proposed diagnostic criteria for subclinical Cushing's syndrome associated with adrenal incidentaloma

Yuko Akehi[1)], Hisaya Kawate[2)], Kunitaka Murase[1)], Ryoko Nagaishi[1)], Takashi Nomiyama[1)], Masatoshi Nomura[2)], Ryoichi Takayanagi[2)] and Toshihiko Yanase[1),2)]

[1)]*Department of Endocrinology and Diabetes Mellitus, School of Medicine, Fukuoka University, Fukuoka 814-0180, Japan*
[2)]*Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan*

Abstract. Subclinical Cushing's syndrome (SCS) associated with adrenal incidentaloma is usually characterized by autonomous cortisol secretion without overt symptoms of Cushing's syndrome (CS). Although the diagnostic criteria for SCS differ among countries, the 1 mg dexamethasone suppression test (DST) is essential to confirm the presence and the extent of cortisol overproduction. Since 1995, SCS has been diagnosed in Japan based on serum cortisol levels ≥3 μg/dL (measured by radioimmunoassay [RIA]) after a 1 mg DST. However, the increasing use of enzyme immunoassays (EIA) instead of RIA has hindered the diagnosis of SCS because of the differing sensitivities of commercially available assays, particularly for serum cortisol levels of around 3 μg/dL. One way to overcome this problem is to lower the cortisol threshold level after a 1 mg DST. In the present study, we examined the clinical applicability of lowering the cortisol threshold to 1.8 μg/dL, similar to the American Endocrine Society's guidelines for CS, by reanalyzing 119 patients with adrenal incidentaloma. Our findings indicate that serum cortisol levels ≥1.8 μg/dL after 1 mg DST are useful to confirm the diagnosis of SCS if both of the following criteria are met: (1) basal ACTH level <10 pg/mL (or poor plasma ACTH response to corticotrophin-releasing hormone) and (2) serum cortisol ≥5 μg/dL at 21:00 to 23:00 h. If only one of (1) and (2) are met, we recommend that other clinical features are considered in the diagnosis of SCS, including serum dehydroepiandrosterone sulfate levels, urine free cortisol levels, adrenal scintigraphy, and clinical manifestation.

Key words: Adrenal incidentaloma, Subclinical Cushing's syndrome, Diagnostic criteria

SUBCLINICAL CUSHING'S SYNDROME (SCS) is defined as an adrenal tumor (usually adenoma) with autonomic cortisol secretion without overt symptoms of Cushing's syndrome (CS). SCS is also referred to as subclinical hypercortisolemia (SH) [1]. Because of the broad variation in autonomic cortisol secretion among patients, diagnostic criteria for SCS have not been fully established. SCS has attracted much attention because it often is masked by lifestyle-related diseases such as diabetes mellitus (DM), metabolic syndrome (MetS), hypertension, and dyslipidemia [1, 2].

In Japan, the diagnostic criteria for SCS were first proposed in 1995 by the Disorders of Adrenal Hormones Research Committee, which was supported by the Ministry of Health, Labour and Welfare, Japan (Table 1) [3]. The diagnosis of SCS involves three essential features: (1) the presence of an adrenal mass, (2) the absence of characteristic features of CS, and (3) basal serum cortisol levels in the normal range with no suppression of serum cortisol (*i.e.*, ≥3.0 μg/dL) after a low-dose (1 mg) dexamethasone suppression test (DST) performed overnight. The cortisol threshold level of ≥3 μg/dL after a 1 mg DST was established based on the finding that among 30 patients with non-functioning adrenal adenoma, all of them had cortisol levels <3 μg/dL after a 1 mg DST [3] measured by a radioimmunoassay (RIA). In addition to fulfilling these criteria, at least one of the criteria listed in Table 1 (3.3. to 3.7.) is required for the final diagnosis of SCS. These additional criteria include suppressed plasma ACTH levels in the early morning (<10 pg/mL) and/or a decreased ACTH response to corticotropin-releasing hormone (CRH) stimulation, unilateral uptake by adrenal scintigraphy, lack of diurnal changes in serum cortisol levels, serum dehydroepi-

Submitted Dec. 22, 2012; Accepted Mar. 15, 2013 as EJ12-0458
Released online in J-STAGE as advance publication Apr. 10, 2013

Correspondence to: Toshihiko Yanase, M.D., Department of Endocrinology and Diabetes Mellitus, School of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. E-mail: tyanase@fukuoka-u.ac.jp

Table 1 Diagnostic criteria for adrenal SCS proposed by the Research Committee on Disorders of Adrenal Hormones with the Ministry of Health, Labour and Welfare, Japan, in 1995 [3]

1. Presence of an adrenal mass (adrenal incidentaloma)
2. Lack of characteristic features of CS[1]
3. Laboratory data:
   - 3.1. Normal basal serum cortisol levels[2]
   - 3.2. Autonomic cortisol secretion confirmed by an overnight 1 mg DST[3]
   - 3.3. Low plasma ACTH levels in the early morning[4]
   - 3.4. Unilateral uptake on adrenal scintigraphy
   - 3.5. No diurnal changes in serum cortisol levels
   - 3.6. Low serum DHEA-S levels[5]
   - 3.7. Transient adrenal insufficiency or atrophy of the residual normal adrenal after removing the adrenal tumor

The diagnosis is defined based on the presence of 1, 2, 3.1, and 3.2. plus at least one 3.3.–3.7.

[1] Hypertension, obesity and glucose intolerance are not regarded as characteristic conditions of CS.
[2] Serum cortisol should be measured at least twice.
[3] SCS is suspected if the serum cortisol level is ≥3.0 μg/dL after an overnight 1 mg DST and is considered very likely if the serum cortisol level is ≥1.0 μg/dL after an overnight 8 mg DST.
[4] Basal ACTH level <10 pg/mL or poor ACTH response to CRH.
[5] DHEA-S level lower than the age- and sex-matched reference level.
CS, Cushing's syndrome; DHEA-S, dehydroepiandrosterone sulfate; DST, dexamethasone suppression test; SCS, subclinical Cushing's syndrome; CRH, corticotropine-releasing hormone

androsterone sulfate (DHEA-S) level below the age- and sex-matched reference level, and transient adrenal insufficiency or atrophy of the residual normal adrenal after removal of the adrenal tumor.

The changes in cortisol assay methods over the last decade have prompted the question of whether the present threshold value (*i.e.*, ≥3 μg/dL after an overnight 1 mg DST) is appropriate for initial screening of SCS. Enzyme immunoassays (EIA) are now more widely used than RIAs, which were the primary assay method used at the time the initial criteria were established. However, the sensitivities of these assay kits vary considerably [4]. The different sensitivity of assay kits for low serum cortisol levels, particularly in the range of 1–3 μg/dL sometimes results in false-positive or false-negative diagnosis of SCS. Because cortisol levels ≥1 μg/dL after a high-dose, 8 mg, DST are required in the current diagnostic criteria for SCS, discrepancies in the results of DSTs, including suppression after a 1 mg DST but no suppression after a 8 mg DST have been reported [5]. One way to overcome this problem is to standardize cortisol levels among the many assay kits based on the determination of an absolute cortisol value measured by liquid chromatography/tandem mass spectrometry [6]. Although this process is currently underway in Japan, it may be some time before it is implemented in medical practice.

In 2008, the American Endocrine Society published their guidelines for CS, including Cushing's disease (CD), in 2008 [7]. They recommended that the lower threshold for the cortisol level after an overnight 1 mg DST should be lowered to <1.8 μg/dL (50 nmol/L) [7], a level that increases the sensitivity to >95% [8]. Therefore, another method to overcome assay variation at low cortisol levels is to simply lower the cortisol threshold level to 1.8 μg/dL after a 1 mg DST. Although this change in threshold shows high sensitivity, it also decreases the specificity of the diagnosis. Therefore, to increase the specificity of the diagnosis, it will be necessary to use this criterion in combination with other criteria to accurately diagnose SCS. Accordingly, in the present study, we reevaluated the diagnosis of SCS in 119 patients with adrenal incidentaloma and sought to establish new diagnostic criteria for SCS based on a lower serum cortisol threshold (*i.e.*, 1.8 μg/dL instead of 3 μg/dL) after a 1 mg DST in combination with other clinical parameters.

Materials and Methods

Subjects

The study consisted of 119 patients (69 women and 50 men) aged 24–78 years old (58.8 ± 11.9 years, mean ± SD) who were hospitalized at Fukuoka University Hospital or Kyushu University Hospital between April 2003 and August 2011 to undergo further examinations of incidentally detected adrenal masses and who were finally diagnosed with SCS or non-functioning adrenal

adenoma. All of the incidentalomas were discovered by abdominal ultrasonography, computed tomography, or magnetic resonance imaging performed to evaluate another unrelated disease. Patients with pheochromocytoma and primary aldosteronism were excluded from the study because their urine catecholamine levels, metabolite levels, and plasma aldosterone concentration/plasma renin activity ratio were normal. In borderline patients, primary aldosteronism was ruled out after a furosemide-upright test, captopril test, and ACTH test. All the patients lacked overt signs or symptoms of CS. Overall, 22/119 patients (18.5%) were diagnosed with SCS based on the Japanese criteria for SCS [3]. In this study, we reevaluated these 22 patients with SCS and the other patients with non-functioning adrenal adenoma using the new cortisol threshold of ≥1.8 μg/dL after an overnight 1 mg DST. The study protocol was approved by the Institutional Review Boards at Fukuoka University Hospital and Kyushu University Hospital.

Methods

We divided the subjects into six groups according to serum cortisol levels after an overnight 1 mg DST, as follows: 0 ~ <1.0, ≥1.0 ~ <1.8, ≥1.8 ~ <3.0, ≥3.0 ~ 4.0, ≥4.0 ~ 5.0 or ≥5 μg/dL. The patients were also divided into two groups based on serum cortisol levels of <1.8 μg/dL or ≥1.8 μg/dL after a 1 mg DST. Additional criteria included: (i) serum DHEA-S level below the age- and sex-matched reference level [9]; (ii) 24-h urinary free cortisol (UFC) level ≥70 μg/day [10]; (iii) basal ACTH level <10.0 pg/mL [3, 11]; (iv) serum cortisol level ≥5.0 μg/dL at 21:00 to 23:00 h [3, 12]; and (v) fulfillment of both iii and iv. To determine whether these criteria were associated with the unfavorable clinical conditions caused by hypercortisolemia, we examined the prevalence of hypertension, glucose intolerance (impaired glucose tolerance [IGT] or DM), and hypercholesterolemia, in patients who fulfilled criteria iii to v. Hypertension was defined as systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg according to the guidelines of the Japan Society of Hypertension 2004 [13]. Glucose intolerance (IGT or DM) was defined as findings of 75g glucose tolerance test or fasting plasma glucose levels ≥110 mg/dL or occasional plasma glucose levels ≥200 mg/dL according to the World Health Organization 1999 criteria [14]. Hypercholesterolemia was defined as serum LDL-C levels ≥140 mg/dL, as calculated using Friedewald's formula [15]. Patients treated with medications for hypertension, IGT/DM, or hypercholesterolemia were also defined as having these diseases. In both institutions, serum cortisol and plasma ACTH levels were measured using an electrochemiluminescence immunoassay (ECLIA) (Elecsys 2010; Roche, Mannheim, Germany) [16, 17]. The detection limits for plasma ACTH and cortisol are 1 pg/mL and 0.018 μg/dL, respectively. The interassay coefficient variance of ACTH was 1.6-3.6 %. The intrassay coefficient variance of ACTH and cortisol was 0.5-4.2 % and <15 %, respectively.

Statistical analysis

In all analyses, values of $p < 0.05$ were considered statistically significant. The Mann–Whitney U test was used to compare age and body mass index (BMI) between subjects divided according to serum cortisol levels of <1.8 *vs*. ≥1.8 μg/dL after a 1 mg DST. The Fisher's analysis was used to compare the prevalence of hypertension, IGT/DM, and hypercholesterolemia between these two groups. Univariate and multivariate analysis using multiple regression analysis were used to identify factors associated with serum cortisol levels after a 1 mg DST. The univariate and multivariate analysis revealed that the gender difference and IGT/DM were the only factors to be independently associated with the cortisol value after a 1 mg DST. The cut-off value for IGT and DM was determined by stepwise discriminant analysis. The sensitivity, specificity, and accuracy of the criteria used to detect IGT/DM were also calculated using discriminant analysis. All statistical evaluations were done according to the suggestions of a specialist of statistics.

Results

Distribution of patients according to disease status and serum cortisol levels

The distribution of patients according to cortisol levels after a 1 mg DST (*i.e.*, 0 ~ <1.0, ≥1.0 ~ <1.8, ≥1.8 ~ <3.0, ≥3.0 ~ 4.0, ≥4.0 ~ 5.0, and ≥5 μg/dL are shown in Fig. 1A–E. Very few patients in each category of serum cortisol had serum DHEA-S levels below the normal range. Only 27.0% (n=7/26) of patients with serum cortisol ≥1.8 μg/dL had low DHEA-S levels. These results suggest that DHEA-S has limited diagnostic relevance, even at a cortisol level of ≥1.8 μg/dL after a 1 mg DST (Fig. 1A).

When we used UFC ≥70 μg/day as a candidate criterion, a relatively high proportion (61.5%) of patients

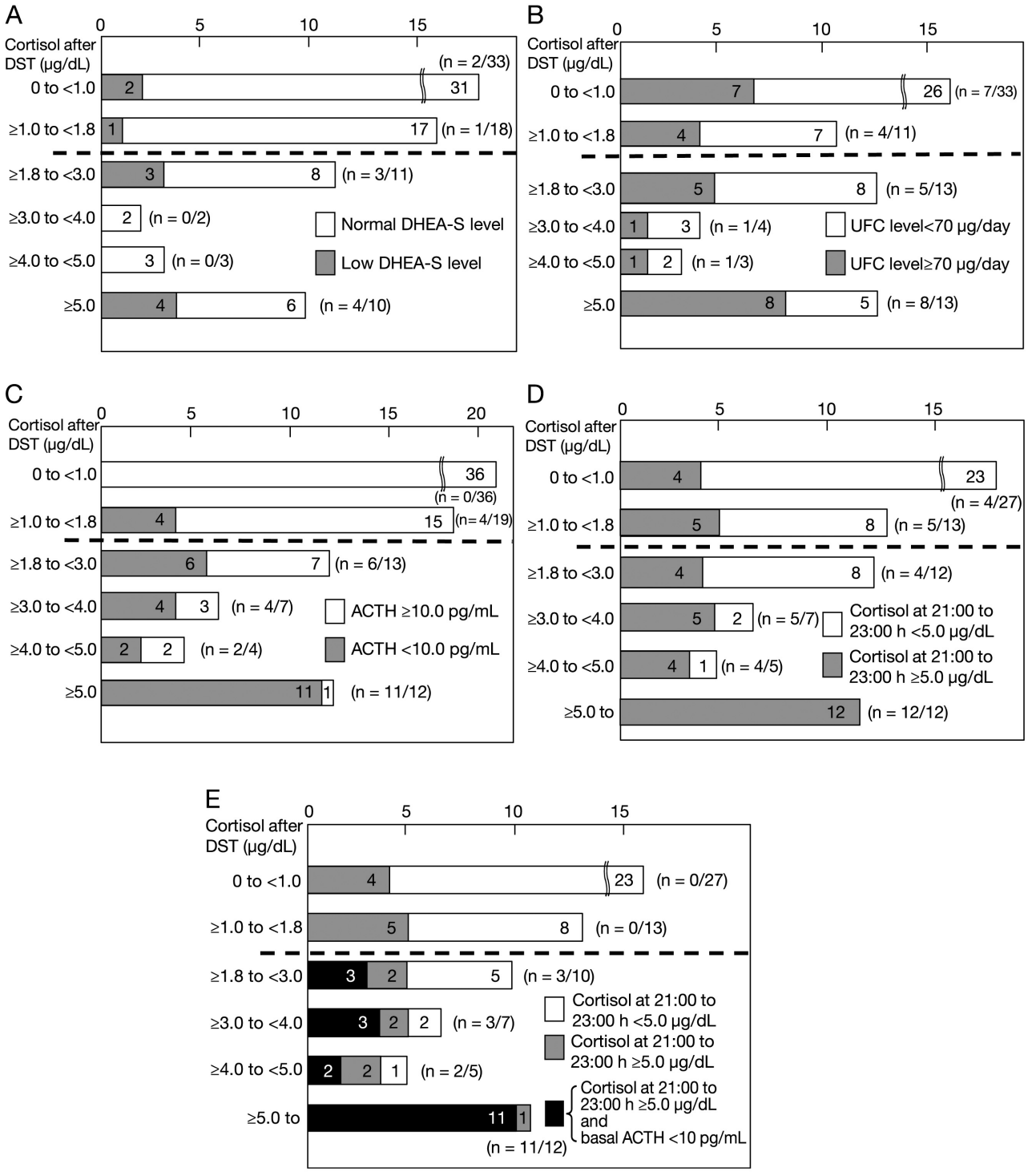

Fig. 1 Classification of patients according to hormonal characteristics and serum cortisol levels after a 1 mg DST
(A) Proportion of patients with serum DHEA levels below the age- and sex-matched reference level [9]. (B) Proportion of patients with UFC levels ≥70 μg/day or <70 μg/day. (C) Proportion of patients with basal ACTH levels ≥10 pg/mL or <10 pg/mL. (D) Proportion of patients with serum cortisol levels ≥5 μg/dL or <5 μg/dL at 21:00 to 23:00 h. (E) Proportion of patients with basal ACTH levels <10.0 pg/mL and serum cortisol levels ≥5 μg/dL at 21:00 to 23:00 h. The patients were divided according to serum cortisol levels after a 1 mg DST into six categories: 0 ~ <1.0, ≥1.0 ~ <1.8, ≥1.8 ~ <3.0, ≥3.0 ~ 4.0, ≥4.0 ~ 5.0, or ≥5 μg/dL. The figures are also split by dot line according to serum cortisol levels of <1.8 μg/dL and ≥1.8 μg/dL.
The number of the cross-axis line indicates the number of the patients.

with cortisol levels ≥ 5 μg/dL (8/13) fulfilled this criterion. Although UFC was ≥70 μg/day in 45.4% (n=15/33) of patients with serum cortisol ≥1.8 μg/dL, UFC did not seem to be associated with autonomic cortisol secretion, instead reflecting nonspecific individual variation (Fig. 1B).

None of the patients with serum cortisol levels of 0 ~ <1.0 μg/mL but 11/12 subjects with serum cortisol ≥5.0 μg/dL had basal ACTH levels <10.0 pg/mL. Overall, 63.9% (n=23/36) and 73.9% (n=17/23) of patients with serum cortisol levels of ≥1.8 μg/dL and ≥3.0 μg/dL, respectively, met this criterion. Therefore, ACTH <10.0 pg/mL seems to be a relatively strong marker for autonomic cortisol secretion. However, it is important to note that in patients with borderline cortisol levels, 4/19 patients with cortisol levels of ≥1.0 ~ <1.8 μg/dL met this criterion whereas 7/13 patients with cortisol levels of ≥1.8 ~ <3.0 did not (Fig. 1C). These results indicate that a basal ACTH level of <10.0 pg/mL is a poor diagnostic factor for SCS among patients with a serum cortisol level of <5 μg/dL after a 1 mg DST.

When we included serum cortisol level ≥5.0 μg/dL at 21:00 to 23:00 h as a possible diagnostic parameter, all of the subjects with serum cortisol levels ≥5.0 μg/dL after a 1 mg DST met this criterion. Overall, 69.4% (n=25/36) and 87.5% (n=21/24) of patients with serum cortisol levels of ≥1.8 μg/dL and ≥3.0 μg/dL met this criterion, respectively, indicating that it is a strong marker for autonomic cortisol secretion (Fig. 1D).

Finally, we assessed the diagnostic potential of combining criteria, namely simultaneous satisfaction of both basal ACTH level <10.0 pg/mL and serum cortisol ≥5.0 μg/dL at 21:00 to 23:00 h). In this analysis (Fig. 1E), none of the patients with serum cortisol levels <1.8 μg/dL after 1 mg DST fulfilled both criteria, indicating that this combination rules out subjects with normal cortisol autonomy.

Taken together, these results indicate that two criteria, namely basal ACTH level <10.0 pg/mL and serum cortisol level ≥5.0 μg/dL at 21:00 to 23:00 h, are useful markers that increase the specificity of the diagnosis of SCS, when used either alone or in combination. Therefore, we considered these in our proposed diagnostic criteria for SCS.

Prevalence of hypertension, IGT/DM, and hypercholesterolemia

To examine the clinical significance of the two criteria established above, we examined the associations between these criteria and other clinical disorders (Table 2). In our initial comparison according to serum cortisol levels after a 1 mg DST, we found no differences in age, BMI, or the prevalence rates of hypertension, IGT/DM, and hypercholesterolemia between patients with serum cortisol levels of <1.8 μg/dL *vs*. ≥1.8 μg/dL. However, among patients with basal ACTH levels of <10.0 pg/mL, the prevalence of IGT/DM was significantly higher in patients with serum cortisol ≥1.8 μg/dL compared with patients with serum cortisol <1.8 μg/dL (66.8% *vs*. 41.8%). The prevalence of IGT/DM was also significantly higher in patients with serum cortisol ≥1.8 μg/dL who meet both basal ACTH levels of <10.0 pg/mL and serum level of cortisol at 21:00 to 23:00 h ≥5 μg/dL compared with patients with serum cortisol <1.8 μg/dL (77.8% *vs*. 41.8%). Taken together, IGT/DM seems to be the most sensitive clinical manifestation associated with subtle cortisol overproduction in patients with SCS.

Table 2 Prevalence rates of hypertension, glucose intolerance, and hypercholesterolemia in patients stratified by each of the criteria

	age	BMI (kg/m^2)	Hypertension	IGT/DM	Hypercholesterolemia
Serum cortisol after 1 mg DST					
<1.8μg/dL (n=79)	58±13	24.9±4.1	42/79 (53.1%)	33/79 (41.8%)	32/79 (40.5%)
≥1.8μg/dL (n=40)	60±10.3	25.0±4.0	21/40 (52.5%)	22/40 (55.0%) *	* 16/40 (40.0%)
Under the condition of serum cortisol ≥ 1.8μg/dL after 1mg DST					
Basal ACTH level <10 pg/mL (n=24)	60±9.9	25.0±3.5	13/24 (54.2%)	16/24 (66.8%)	12/24 (50.0%)
F at night ≥5μg/dL (n=24)	58±10.6	24.0±3.2	13/24 (54.1%)	15/24 (62.5%)	12/24 (50.0%)
Basal ACTH level <10 pg/mL & F at night ≥5μg/dL (n=18)	59±10.4	25.0±3.0	10/18 (55.6%)	14/18 (77.8%)	10/18 (55.6%)

BMI, Body mass index; IGT, impaired glucose tolerance; DM, diabetes mellitus DST, Dexamethasone suppression test; F at night, serum cortisol level at 21:00 to 23:00 h

*p <0.05, The values are indicated as Mean ±SD.

Table 3 Univariate analysis of several parameters associated with serum cortisol level after 1mg DST

	Sample number	Regression Coefficient	Correlation Coefficient	*p*-value
Gender difference	119	-1.62	-0.27	0.0032
Age	119	0.007	0.03	0.7686
Hypertension	119	-0.591	-0.13	0.2835
IGT/DM	119	1.731	0.32	0.0012
Hypercholesterolemia	119	-0.301	0.11	0.3018
BMI	119	0.025	0.03	0.7874

Gender difference and IGT/DM were the only significant parameters.
IGT/DM, impaired glucose tolerance/diabetes mellitus; BMI, body mass index

Table 4 Multivariate analysis of several parameters associated with serum cortisol level after 1mg DST

	Regression Coefficient	S.E.	*p*-value
Gender difference	-0.249	0.54	0.0149
IGT/DM	0.268	0.52	0.0035

The R^2 value for the multiple regression model was 0.41, which indicates a good statistical model. Gender difference and IGT/DM were the only significant parameters.
IGT/DM, impaired glucose tolerance/ diabetes mellitus; S.E., standard error

Table 5 The cut-off value of serum cortisol level (μg/dL) after 1mg DST to differentiate the presence of IGT/DM

IGT/DM	number	mean	S.D.	variance	cut-off value of cortisol
no	n=64	1.35	1.52	2.29	1.83
yes	n=55	3.39	4.29	18.43	

The calculation was done by stepwise discriminate analysis.
The serum cortisol value was μg/dL.
DST, dexamethasone suppression test; IGT/DM, impaired glucose tolerance/ diabete mellitus; S.D., standard deviation

Table 6 The sensitivity, specificity and accuracy of each criteria to detect IGT/DM

	Sensitivity	Specificity	Accuracy	*p*-value
(1) cortisol after 1mg DST≥1.8μg/dL	81.3	48	61.1	0.0118
(2) ACTH<10.0 pg/mL	86.4	52.4	69.8	0.0019
(3) cortisol at 21:00 to 23:00 h ≥5.0μg/dL	68.8	52.6	60.1	0.0142
(1) & (2)	71.4	58.8	62.5	0.1994
(1) & (3)	86.3	60.8	70.6	0.1226
(1) & (2) & (3)	85.7	72.7	77.8	0.0382

The analysis was done by discriminate analysis.

The serum cortisol level after a 1 mg DST is associated with IGT/DM

We performed univariate and multivariate analysis using multiple regression analysis to identify clinical parameters that are associated with the serum cortisol level after a 1 mg DST (Tables 3 and 4). Both analyses revealed that the serum cortisol level after a 1 mg DST was significantly associated with sex and the presence of IGT/DM. The R^2 value for the multiple regression model in multivariate analysis was 0.41, which indicates a very good statistical model. The gender difference indicates that the mean value of serum cortisol level after 1mg DST was greater in women than men (2.82±3.61 *vs.* 1.20±1.39, mean ± S.D.). A strong association between the serum cortisol level after a 1 mg DST and IGT/DM ($p = 0.0035$) by multivariate analysis (Table 4) suggests that IGT/DM is an independent strong factor related with the serum cortisol level after a 1 mg DST. Furthermore, the cut-off value for the serum cortisol level after a 1 mg DST to determine the presence of IGT/DM was 1.83 μg/dL based on discriminant analysis (Table 5), which is very similar to the proposed cut-off value of 1.8 μg/dL.

Proposed diagnostic criteria for SCS statistically discriminates IGT/DM with good accuracy

The sensitivity, specificity, and accuracy of each criterion and the combined criteria for the detection of IGT/DM were calculated using discriminant analysis (Table 6). These results indicate that the combination criteria of serum cortisol level ≥1.8 μg/dL after a 1 mg DST, ACTH<10 pg/mL and serum cortisol level ≥5.0 μg/dL at 21:00 to 23:00 h show high sensitivity, high specificity and high accuracy with significance for the detection of IGT/DM.

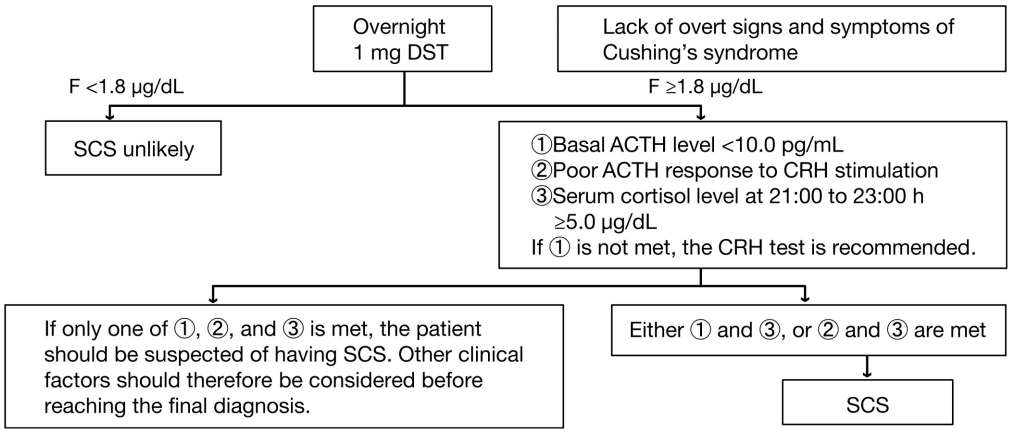

Fig. 2 Proposed algorithm for the diagnosis of SCS
In our algorithm, patients with serum cortisol levels ≥1.8 μg/dL after a 1 mg DST should also have a basal ACTH level <10 pg/mL and/or cortisol level ≥5 μg/dL at 21:00 to 23:00 h to fulfill the diagnostic criteria for SCS. If both of these additional criteria are met, SCS is very likely. In cases with normal ACTH levels, the absence or a poor ACTH response to CRH should be examined. If only one of the three additional criteria are met, the patient should be suspected of having SCS. The consulting physician should consider other factors that are commonly used for the diagnosis of SCS, including serum DHEA-S levels, urine free cortisol, adrenal scintigraphy, and clinical manifestations before reaching the final diagnosis.

Proposed diagnostic algorithm for SCS (Fig. 2)

Because none of the patients with serum cortisol levels of <1.8 μg/dL after a 1 mg DST had basal ACTH levels of <10.0 pg/mL and serum cortisol levels ≥5.0 μg/dL at 21:00 to 23:00 h, we were able to incorporate these three criteria into a diagnostic algorithm, as well as poor responses to CRH stimulation. However, it must be noted that two patients with a basal ACTH level ≥10 pg/mL showed a poor plasma ACTH response, with an increase in ACTH of <2-fold. Their basal ACTH levels were 21.3 pg/mL and 17.8 pg/mL, and reached peak values of 28.2 pg/mL (1.32 fold) and 28.6 pg/mL (1.61 fold), respectively, after CRH stimulation. In these patients, the cortisol level after a 1 mg DST were 15.8 μg/dL and 4.2 μg/dL, respectively while the basal cortisol levels at 21:00 to 23:00 h were 16.5 μg/dL and 8.1 μg/dL, respectively, supporting the diagnosis of SCS. Clearly, the findings in these patients suggest that basal ACTH levels alone are not necessarily sufficient to evaluate ACTH suppression. Consequently, we suggest that a poor ACTH response to CRH stimulation should be included as one of the diagnostic criteria for SCS.

In our algorithm, patients with cortisol levels ≥1.8 μg/dL after a 1 mg DST should also have a basal ACTH level <10 pg/mL and/or cortisol level ≥5 μg/dL at 21:00 to 23:00 h to fulfill the diagnostic criteria for SCS. If both of these additional criteria are met, SCS is very likely. In cases with normal ACTH level, no or little ACTH response to CRH should be carefully examined. If only one of the three additional criteria are met, the consulting physician should consider other factors commonly used for the diagnosis of SCS, including serum DHEA-S levels, urine free cortisol, adrenal scintigraphy, and clinical manifestations. After all, two cases previously judged as having non-functioning tumor by the conventional criteria were newly diagnosed as having SCS by the proposed criteria. On the other hand, among 22 cases previously judged as having SCS by the conventional criteria, 4 cases were changed to the diagnosis of suspected SCS by the proposed criteria. By using the proposed criteria, 20/119 patients with adrenal incidentalomas were diagnosed with definite SCS.

Discussion

SCS occurs in 5–30% of patients with an adrenal incidentaloma [18, 19] and in 0.2–2% of the general adult population [20]. A recent nationwide survey conducted in Japan between 2003 and 2007 revealed an incidence of 1829 patients with SCS over 5 years [21], whereas a survey done almost 10 years earlier (1992-1996) revealed an incidence of only 290 patients/5 years [22]. Clearly, clinicians in Japan are becoming more aware of SCS as a disease. This increasing awareness can be attributed to the Japanese guidelines for SCS published in 1995. This is clinically important because SCS is associated with cardiovascular risk factors, including MetS, IGT, DM, hypertension, and hyperlipidemia.

Many parameters have been proposed to detect cortisol overproduction, but each parameter has some advantages and disadvantages. Briefly, while 24 h measurement of UFC is advantageous in that the value is not affected by corticosteroid binding globulin (CBG), the values show high interday variation [7, 23]. Measurement of salivary cortisol levels in the late evening or at midnight is very useful for the diagnosis of CS and is not affected by CBG. However, this parameter may show poor sensitivity for the diagnosis of SCS [23-25]. As shown in the present study, serum cortisol at late at night (21:00 to 23:00 h) is a particularly useful factor for the diagnosis of CS and SCS, but this must be measured in-hospital. An overnight 1 mg DST is the most commonly used test for the diagnosis of CS or SCS and may be performed either alone or in combination with other tests. A widely used threshold value for serum cortisol after an overnight 1 mg DST is <5 μg/dL (<140 nmol/L) [1, 7]. However, because of the low sensitivity and high specificity of this value, false-negative results are obtained in some patients with CD or SCS. Consequently, some experts, including the American Endocrine Society [7, 8], have recommended a lower cortisol threshold of <1.8 μg/dL (50 nmol/L) after an overnight 1 mg DST, as this level increases diagnostic sensitivity. Therefore, in the present study, we tried to establish new diagnostic criteria for SCS using serum cortisol levels of ≥1.8 μg/dL after a 1 mg DST, consistent with the American Endocrine Society guidelines [7]. Here, we showed that it is possible to diagnose SCS using this lower threshold in combination with other factors, including a basal ACTH level of <10 pg/mL, poor ACTH response to CRH stimulation, and cortisol level ≥5 μg/dL at 21:00 to 23:00 h.

Two patients included in this study with DM and hypertension who had been diagnosed with non-functioning adenomas using conventional diagnostic criteria was newly diagnosed with SCS when we applied our new criteria, as their serum cortisol level was 2.2 and 1.8 μg/dL after a 1 mg DST, their basal ACTH levels were 5.0 and 6.6 pg/mL, and their cortisol levels were 8.3 and 5.5 μg/dL at 21:00 to 23:00 h, respectively. Therefore, the lower cortisol threshold level of 1.8 μg/dL rather than 3.0 μg/dL after a 1 mg DST seems to be more appropriate for better identifying patients with SCS. On the other hand, 4 cases were changed to the diagnosis of suspected SCS by the proposed criteria. These four patients had serum cortisol ≥1.8 μg/dL after a 1 mg DST (4.7, 10.7, 3.1 and 4.6 μg/dL) and cortisol level ≥5 μg/dL at 21:00 to 23:00 h (5.0, 6.9, 5.4 and 7.4 μg/dL), but basal ACTH was ≥10 pg/mL (32.0, 10.3, 23.1 and 42.0 pg/mL, respectively). As the CRH test was not performed in these four patients, we could not exclude the possibility of SCS. Therefore, these patients should be considered to have suspected SCS until further diagnostic tests can be performed. Clinically, the adrenal tumors in these four patients are unlikely to be functional because they did not have any metabolic abnormalities (*i.e.*, IGT/DM), and only two had hypertension.

In addition, the proposed threshold level is technically appropriate because a previous study showed limited reproducibility of serum cortisol levels in the range of 2–4 μg/dL with a range of assay kits [4]. Therefore, the cut-off value of 1.8 μg/dL after a 1 mg DST may be favorable in terms of globalization of the diagnostic criteria for SCS or subclinical hypercortisolemia (SH).

One limitation of our study is that CRH test was performed in just six patients, including four patients with SCS, out of 119 patients. Therefore, we could not evaluate the statistical or clinical relationship between cortisol levels after a 1 mg DST and the ACTH response to CRH in the present study. Of four patients diagnosed with SCS using the proposed algorithm, two had basal ACTH levels ≥10 pg/mL and their ACTH response to CRH was poor, providing strong support for the diagnosis of SCS, as described in the Results session. In another two patients with SCS, their ACTH levels were 5.2 and 6.6 pg/mL, and increased by >2-fold to peak values of 10.5 and 28.6 pg/mL, respectively, following CRH stimulation. A relatively good ACTH response to CRH stimulation, despite basal ACTH levels <10 pg/mL, in patients with SCS was also reported by other researchers [26]. These findings suggest that, depending on the extent of autonomic cortisol production, SCS may progress from a stage associated with suppression of basal ACTH levels to a stage in which the ACTH response to CRH is suppressed.

To examine the clinical relevance of the proposed diagnostic criteria for SCS, we compared the prevalence of hypertension, IGT/DM, and hypercholesterolemia between groups of patients fulfilling each of the criteria. We confirmed that the prevalence of IGT/DM in patients meeting both of these criteria was significantly higher than that in the control group (77.8% *vs.* 41.8%), but the prevalence of hypertension and hypercholesterolemia was not. The results suggest that these two criteria could identify patients with other diseases

associated with hypercortisolism, and are useful and appropriate for diagnosing SCS. In earlier studies, the prevalence rates of DM, hypertension, and hypercholesterolemia in patients with SCS were 36–65%, 45–91.6%, and 50–71%, respectively [2, 27-29]. The different prevalence rates of these diseases among these studies might be because of the application of different diagnostic criteria for SCS. Nevertheless, we should consider that the markers for cortisol secretion were derived from cross-sectional studies and they may not reflect the longitudinal biological effects of cortisol over the duration of the disease. Unfortunately, it is not possible to precisely estimate the duration of the disease because of the lack of specific clinical manifestations. The sensitive and specific detection of IGT/DM using the serum cortisol level after a 1 mg DST in our subjects was further supported by the results of univariate and multivariate analyses of several clinical parameters. Interestingly, the cut-off value for the serum cortisol level after a 1 mg DST for the detection of IGT/DM was coincidentally 1.83 μg/dL. The sensitivity, specificity, and accuracy of the proposed diagnostic criteria for the detection of IGT/DM were also statistically validated. More functional SCS may be possible to pick up by the proposed new criteria.

Few studies have examined the benefits of surgery in patients with SH. Nevertheless, most of the studies that have been performed agree that adrenalectomy normalizes the endocrine abnormalities associated with SH, and there is some evidence for the amelioration of metabolic disorders [2, 30-32]. Indeed, significant decreases in systolic blood pressure were consistently reported in these studies. To our surprise, even in patients without SH before surgery, blood pressure improved after surgery [25, 30]. However, it is still not possible to determine whether this phenomenon is a natural change or reflects subtle autonomic cortisol secretion, even in non-functioning adenoma. Therefore pursuing the operative indications for adrenal incidentalomas may be an important target of research in future studies.

In conclusion, we reevaluated the current diagnostic criteria for SCS, and developed new criteria by first lowering the cortisol threshold from 3 μg/dL to 1.8 μg/dL after a 1 mg DST. Based on our present results, we propose that serum cortisol ≥1.8 μg/dL after 1 mg DST together with basal ACTH level <10 pg/mL (or poor plasma ACTH response to CRH stimulation) and serum cortisol ≥ 5 μg/dL at 21:00 to 23:00 h are appropriate diagnostic criteria for SCS. However, for patients with serum cortisol ≥1.8 μg/dL and the presence of only one of the criteria, we recommend that other findings are considered, including serum DHEA-S level, urine free cortisol, adrenal scintigraphy, and clinical manifestations, before reaching a diagnosis of SCS.

Acknowledgements

This research was partially supported by Intractable Disease Research Grant of Ministry of Health, Labour and Welfare, Japan (ID: 11103517).

Conflicts of Interest

The authors declare no conflict of interest relevant to this manuscript.

References

1. Chiodini L (2011) Diagnosis and Treatment of Subclinical Hypercortisolism. *J Clin Endocrinol Metab* 96: 1223-1236.
2. Tauchmanovà L, Rossi R, Biondi B, Pulcrano M, Nuzzo V, et al. (2002) Patients with subclinical Cushing's syndrome due to adrenal adenoma have increased cardiovascular risk. *J Clin Endocrinol Metab* 87: 4872-4878.
3. Nawata H, Demura H, Suda T, Takayanagi R (1996) Adrenal preclinical Cushing's syndrome, Annual report of the Ministry of Health and Welfare "Disorder of Adrenal Hormones" Research Committee, Japan 1995: 223-226 (In Japanese).
4. Odagiri E, Naruse M, Terasaki K, Yamaguchi N, Jibiki K, et al. (2004) The diagnostic standard of preclinical Cushing's syndrome: evaluation of the dexamethasone suppression test using various cortisol kits. *Endocr J* 51: 295-302.
5. Katabami T, Obi R, Shirai N, Naito S, Saito N (2005) Discrepancies in results of low- and high-dose dexamethasone suppression tests for diagnosing preclinical Cushing's syndrome. *Endocr J* 52: 463-469.
6. Kawaguchi K, Kuwa K, Takatsu A, Tani W, Kobayashi T (2012) Standarization of cortisol measurement and results of technical examination of low range of cortisol measurement. *ACTH RELATED PEPTIDES* 22: 2-8 (In Japanese).

7. Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, et al. (2008) The diagnosis of Cushing's syndrome: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* 93: 1526-1540.
8. Wood PJ, Barth JH, Freedman DB, Perry L, Sheridan B. (1997) Evidence for the low dose dexamethasone suppression test to screen for Cushing's syndrome--recommendations for a protocol for biochemistry laboratories. *Ann Clin Biochem* 34 (Pt3): 222-229.
9. Bencsik Z, Szabolcs I, Kovács Z, Ferencz A, Vörös A, et al. (1996) Low dehydroepiandrosterone sulfate (DHEA-S) level is not a good predictor of hormonal activity in nonselected patients with incidentally detected adrenal tumors. *J Clin Endocrinol Metab* 81: 1726-1729.
10. Kratz A, Ferraro M, Sluss PM, Lewandrowski KB. (2004) Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Laboratory reference values. *N Engl J Med* 351(23): 1548-1563.
11. Morelli V, Masserini B, Salcuni AS, Eller-Vainicher C, Savoca C, et al. (2010) Subclinical hypercortisolism: correlation between biochemical diagnostic criteria and clinical aspects. *Clin Endocrinol (Oxf)* 73: 161-166.
12. Orth DN (1995) Cushing's syndrome. *N Engl J Med* 332(22): 791-803.
13. The Japanese Society of Hypertension (2009) Guidelines for the management of hypertension (JSH 2009) (In Japanese).
14. World Health Organization (2006) Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation. Geneva, World Health Org.
15. Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18(6): 499-502.
16. Abe M, Araki S, Hyoki M, Sato R, Takamatsu K, et al. (2007) Fundamental evaluation of ACTH measurement using automated electrochemiluminescence-immunoassay system, ECLusys 2010. *Igaku to Yakugaku* 67: 239-244 (In Japanese).
17. Attached document of ECLusys kit for cortisol (3rd version) (2011) (In Japanese).
18. Terzolo M, Bovio S, Reimondo G, Pia A, Osella G, et al. (2005) Subclinical Cushing's syndrome in adrenal incidentalomas. *Endocrinol Metab Clin North Am* 34: 423-439.
19. Terzolo M, Reimondo G, Bovio S, Angeli A (2004) Subclinical Cushing's syndrome. *Pituitary* 7: 217-223.
20. Reincke M (2000) Subclinical Cushing's syndrome. *Endocrinol Metab Clin North Am* 29: 43-56.
21. Yanase T, Fujieda K, Kajino H, Tanahashi Y, Suzuki S (2011) Epidemiological Investigation of Addison's disease and subclinical Cushing's syndrome in Japan. Annual Report of the Ministry of Health and Welfare "Disorder of Adrenal Hormones" Research Committee, Japan 139-146.
22. Nawata H, Takayanagi R, Nakagawa H, Miura K (1999) Epidemiological Investigation of Disorders of Adrenal Hormones in Japan. Annual Report of the Ministry of Health and Welfare "Disorder of Adrenal Hormones" Research Committee, Japan. 11-55 (In Japanese).
23. Kidambi S, Raff H, Findling JW (2007) Limitations of nocturnal salivary cortisol and urine free cortisol in the diagnosis of mild Cushing's syndrome. *Eur J Endocrinol* 157: 725-731.
24. Masserini B, Morelli V, Bergamaschi S, Ermetici F, Eller-Vainicher C, et al. (2009) The limited role of midnight salivary cortisol in the diagnosis of subclinical hypercortisolism in patients with adrenal incidentalomas. *Eur J Endocrinol* 160: 87-92.
25. Tateishi Y, Kouyama R, Mihara M, Doi M, Yoshimoto T, et al. (2012) Evaluation of salivary cortisol measurements for the diagnosis of subclinical Cushing's syndrome. *Endocr J* 59(4): 283-289.
26. Tatsuno I, Uchida D, Tanaka T, Koide H, Shigeta A, et al. (2004) Vasopressin responsiveness of subclinical Cushing's syndrome due to ACTH-independent macronodular adrenocortical hyperplasia. *Clin Endocrinol (Oxf)* 60(2): 192-200.
27. Tsuiki M, Tanabe A, Takagi S, Naruse M, Takano K (2008) Cardiovascular risks and their long-term clinical outcome in patients with subclinical Cushing's syndrome. *Endocr J* 55: 737-745.
28. Emral R, Uysal AR, Asik M, Gullu S, Corapcioglu D, Tonyukuk V, et al. (2003) Prevalence of subclinical Cushing's syndrome in 70 patients with adrenal incidentalomas: clinical, biochemical and surgical outcomes. *Endocr J* 50: 399-408.
29. Rossi R, Tauchmanova L, Luciano A, Di Martino M, Battista C, et al. (2000) Subclinical Cushing's syndrome in patients with adrenal incidentaloma: clinical and biochemical features. *J Clin Endocrinol Metab* 85(4): 1440-1448.
30. Midorikawa S, Sanada H, Hashimoto S, Suzuki T, Watanabe T (2001) The improvement of insulin resistance in patients with adrenal incidentalomas by surgical resection. *Clin Endocrinol (Oxf)* 54: 797-804.
31. Toniato A, Merante-Boschin I, Opocher G, Pelizzo MR, Schiavi F, et al. (2009) Surgical versus conservative management for subclinical Cushing syndrome in adrenal incidentalomas: a prospective randomized study. *Ann Surg* 249 (3): 388-391.
32. Chiodini I, Morelli V, Salcuni AS, Eller-Vainicher C, Torlontano M, et al. (2010) Beneficial metabolic effects of prompt surgical treatment in patients with an adrenal incidentaloma causing biochemical hypercortisolism. *J Clin Endocrinol Metab* 95 (6): 2736-2745.