Steroid metabolites for diagnosing and predicting clinicopathological features in cortisol-producing adrenocortical carcinoma.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

This study was approved by the Human Research Ethics Committee at Chiba University (approval number: 828)

Consent to participate

Informed consent was obtained from the patient before undergoing all clinical procedures.

Consent for publication

Consent for publication was obtained from the patient.

Availability of data and material

Data are available from the corresponding author upon reasonable request.

Code availability

Not applied.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Sawako Suzuki and Tomoki Minamidate. The first draft of the manuscript was written by Sawako Suzuki and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Abstract

About 60% of adrenocortical carcinomas (ACC) are functional, and Cushing's syndrome is the most frequent diagnosis which has been revealed to have particularly poor prognosis. Since 30% of ACC present steroid hormone-producing disorganization, measurement of steroid metabolites in suspected ACC is recommended. Previous reports demonstrated that steroid hormone precursors or its urine metabolites, using liquid chromatography tandem mass spectrometry (LC-MS/MS) or gas chromatography mass spectrometry (GC-MS) respectively, are useful for distinguishing ACC from adrenocortical adenoma. However, whether steroid metabolites can predict pathological characteristics or prognosis has not been elucidated. Here, we examined 12 serum steroid metabolites using immunoassay, which is a more rapid and less costly method compared to LC-MS/MS, in cortisol-producing ACC and cortisol-producing adenomas (CPA). Further, the correlation of each steroid metabolite to the stage classification and pathological status in ACC was analyzed. Reflected disorganized steroidogenesis, immunoassay revealed all basal levels of steroid precursors were significantly increased in cortisol-producing ACC compared to CPA; especially 17-hydroxypregnenolone (androgen precursor) and 11-deoxycorticosterone (mineralocorticoid precursor) showed large area under ROC curve with high sensitivity and specificity, when setting the cut-off at 1.78 ng/ml and 0.4 mg/ml, respectively. In cortisol-producing ACC, 11-deoxycortisol (glucocorticoid precursor) showed significant positive correlations to predictive prognostic factors of ENSAT classification, while testosterone showed significant positive correlations to Ki67-index in both men and women. In conclusion, measurement of serum steroid metabolites using immunoassay has great potential not only for diagnosis but also for prediction of the clinicopathological features associated with disease prognosis of ACC, as a simple non-invasive method.

Keywords: Adrenocortical carcinoma, Steroid metabolites, Cushing's syndrome, Clinicopathology.

Introduction

Adrenocortical carcinomas (ACC) are rare but aggressive endocrine neoplasms from the adrenal cortex. Endocrinologically, about 60% of adrenal cancers in adult patients are functional. Cushing's syndrome is the most commonly associated endocrine disorder [1, 2] and has been revealed to have particularly poor prognosis by systematic review and meta-analysis [3]. Further, about 30% are responsible for multiple hormone production, including steroid hormone precursors; and this is referred to as disorganized steroidogenesis [4]. Prognosis is poor, and 5-year overall survival is lower than 35% in most studies [5, 6]. Management is multidisciplinary and includes surgical resection, oral mitotane, intravenous chemotherapy, and palliative radiation [7]. Therefore, early diagnosis, accurate staging, and appropriate treatment according to progression prediction are important. Because of limited specificity of imaging tests in making the diagnosis of ACC, the combination assessment of the tumor size (≧65mm) [8], and careful pathological investigation with the assessment of the Weiss score (≧3 out of 9) [8], and Ki67-index (≧10%: high risk) [9] is widely used for ACC diagnosis; and European Network for the Study of Adrenal Tumors (ENSAT) system is used for staging classification of ACC [9]. Both growth marker Ki67 and ENSAT classification are also used as predicting prognostic factors of ACC [9]. However, image-guided adrenal biopsy or unilateral adrenalectomy necessary for pathological diagnosis is invasive and cannot be performed in patients with poor general condition. Additionally, adrenal biopsy is an expensive procedure with a reported rate of nondiagnostic biopsies of 8.7 %, and a complication rate of 2.5% [10]; further with the reported disadvantage of lower accuracy with 70% sensitivity and 98% specificity [11]. Therefore, less invasive and less costly methods for diagnosis and prognostic prediction of ACC, instead of repeat imaging and adrenal biopsy, are desired. Recently, steroid profiling has emerged as a powerful novel diagnostic tool for ACC [11-14]. Previous reports demonstrated that examination of serum steroid metabolites using liquid chromatography tandem mass spectrometry (LC-MS/MS) [12, 13] or urine steroid metabolites using gas chromatography mass spectrometry (GC-MS) [15-19] are useful for distinguishing ACC from adrenocortical adenoma. Furthermore, the ENSAT recommends a biochemical workup for suspected ACC that includes serum cortisol, aldosterone, 17-hydroxyprogesterone, dehydroepiandrosterone sulfate (DHEAS), androstenedione, testosterone, and 17-beta-estradiol (http://www.ensat.org/page-1317312). However, whether steroid metabolites can predict pathological characteristics, staging, or prognosis has not

been elucidated to date. In this study, we compared 12 serum steroid metabolites using immunoassay, which is a more rapid and less expensive method compared to LC-MS/MS, in cortisol-producing ACC and cortisol-producing adenomas (CPA). In addition, 24-hour urine excretion of individual 17-ketosteroids as androgen secretions, were examined as well. Furthermore, we identified steroid metabolites which correlated with pathological findings or clinical parameters related to staging and disease prognosis in cortisol-producing ACC.

Materials and Methods

Study population

This study was approved by the Human Research Ethics Committee at Chiba University (approval number: 828), and all patients provided written informed consent. We retrospectively analyzed steroid profiles in all cortisol-producing ACC and CPA who were admitted to our hospital between 2013 and 2018. The final diagnosis had been ascertained by histology and evidence of metastasis in ACC, and by imaging, biochemical, and clinical follow-up showing no evidence of adrenal tumor growth and metastasis in CPA. Endocrinologically, overt Cushing's syndrome was determined in patients with signs or symptoms of excess hormones, suppressed plasma adrenocorticotropic hormone (ACTH), increased 24-hour urinary free cortisol, and high plasma cortisol that could not be suppressed below 5 μg/dl with overnight dexamethasone at doses of either 1 or 8 mg. Subclinical Cushing's syndrome was determined in patients with no signs or symptoms of excess hormones, subnormal suppression following overnight dexamethasone (1 mg; >1.8 μg/dl), and normal 24-hour urinary free cortisol [20, 21].

Clinical evaluation

We analyzed five important clinicopathological parameters in cortisol-producing ACC: ENSAT classification, Weiss score, Ki67-index derived by pathological immunohistological staining, tumor size, and overall survival. We further examined these five clinical parameters' correlation to steroid metabolites. The ENSAT staging system consists of stages I (T1N0M0), II (T2N0M0), III (T1–2N1M0 or T3–4N0–1M0), and IV (TanyNanyM1, metastatic ACC) [22]. Weiss score is comprised of nine histological criteria: (i) high nuclear grade, (ii) mitotic rate greater than five per 50 high power fields (HPF), (iii) atypical mitotic figures, (iv) eosinophilic tumor cell cytoplasm (greater than 75% tumor cells), (v) diffuse architecture

(greater than 33% of tumor), (vi) necrosis, (vii) venous invasion, (viii) sinusoidal invasion, and (ix) capsular invasion [23]. A tumor is labeled malignant when it meets three or more of these histological criteria [8]. The Ki67-index is evaluated using an immunohistochemical assessment of cell proliferation by the detection of Ki67 antigen in neoplastic cell populations; and a Ki67-index of 10% or more is diagnosed as high risk [9].

Clinical samples and hormonal assays

Serum steroid profiling in basal conditions and 24-hour urine specimens were conducted in all subjects. In basal condition, fasting blood was withdrawn after a 15 minutes rest between 8:00 and 9:00 AM. The day before, 24-hour urine specimens were collected for periods of two to three days, and urinary free cortisol and 17-ketosteroid fractions were measured. Urinary and serum cortisol were measured by radioimmunoassay (RIA). 11-deoxycortisol, 11-deoxycorticosterone, corticosterone, aldosterone, pregnenolone, 17-hydroxypregnenolone, and androstenedione were measured by RIA. Progesterone, 17-hydroxyprogesterone, and testosterone were measured by electrochemical luminescence immunoassay (ECLIA). Urinary 17-ketosteroid fractions were measured by GC-MS. Analysis of samples was completed by a Japanese clinical analytical laboratory (SRL, Inc., Tokyo, Japan).

Statistical analyses

Shapiro-Wilk test showed that the endocrinological data were not normally distributed. Hence, pairwise comparisons were performed using Mann-Whitney U-test. Results were expressed as a median (interquartile ranges) and a value of $P < 0.05$ was considered statistically significant. The Chi-Square statistic is used for testing relationships between categorical variables. Receiver operating characteristics (ROC) curves were generated for steroid metabolites which displayed relatively significant differences between ACC and CPA. The area under the ROC curve (AUC) was also calculated. A perfect classifier has AUC = 1, and a completely random classifier has AUC = 0.5. Sensitivity and specificity were calculated at cut-off values providing the highest sensitivity. Correlation between clinicopathological parameters and individual steroid metabolites in ACC was calculated by Pearson's correlation coefficient (R). R values between 0.7 and 1.0 together with $P < 0.05$ can be considered highly correlated. Statistical analysis was

performed using SPSS Statistics for Windows (SPSS Inc., Chicago, IL, USA).

Results

Patients and Clinical characteristics

We evaluated 7 cortisol-producing ACC patients and 25 CPA patients as shown in **Table 1**. ACC population showed a higher age than CPA population, while gender and cortisol producing ability evaluated by urinary cortisol and dexamethasone suppression test did not differ between two groups. Four of 7 ACC patients and 11 of 25 CPA patients presented with signs of Cushing`s syndrome, and were diagnosed as having overt Cushing`s syndrome. All ACC patients and 80% of CPA patients underwent adrenalectomy, and were pathologically diagnosed. Adrenal tumor size and Weiss score were higher in ACC than CPA. Median Ki67 index was 23 in ACC. There were three ACC in stage II, one in stage III, and three in stage IV at diagnosis. Overall survival of ACC varied from 5 to 60 months.

Serum concentration and urinary excretion of steroid metabolites

Comparison of serum steroid metabolites and urinary metabolites between cortisol-producing ACC and CPA is shown in **Table 2.** The comparison of profiles and outcomes of basal serum and urinary 17-ketosteroids between ACC and CPA are summarized in the steroid pathway diagram (**Fig. 1**). Comparison of basal steroid profiles of ACC and CPA revealed striking differences. Significantly higher levels of glucocorticoid precursors (progesterone, 17-hydroxyprogesterone, and 11-deoxycortisol), mineralocorticoid precursors (11-deoxycorticosterone and corticosterone), androgen precursors (pregnenolone, 17-hydroxypregnenolone, and androstenedione), and DHEAS were observed in ACC than in CPA (**Table 2**). There was no significant difference in glucocorticoid, mineralocorticoid, and androgen itself (**Table 2**). The urine 17-ketosteroid fractions of 11-deoxy-17-ketosteroid (androsterone, etiocholanolone, and dehydroepiandrosterone) derived from androgen precursor including androstenedione and DHEA showed a significant increase in ACC compared to CPA (**Table 2**). These data demonstrated ACC had a feature of steroid disorganization as previously reported, and immunoassay is useful enough for distinguishing ACC from CPA. Next, steroid metabolites were plotted on a ROC curve, and then the area under the ROC (AUC) as well as the most appropriate cut-off values were calculated to classify cortisol-producing ACC or CPA. ROC-analysis demonstrated that 12 steroid metabolites had a sensitivity >85% for

detecting ACC (**Table 3**). The ROC curves for 17-hydroxypregnenolone, androstenedione, and 11-deoxycorticosterone were found to have large AUC (0.954, 0.928, and 0.909, respectively) (**Table 3** and **Fig. 2**). Setting the cut-off at 1.78 ng/ml for 17-hydroxypregnenolone revealed 100% sensitivity and 88% specificity (**Table 3**). 11-deoxycorticosterone showed 86% sensitivity and 96% specificity at a cut-off value of 0.4 mg/ml (**Table 3**). Whereas, androstenedione had low specificity with 100% sensitivity and 64% specificity at a cut-off value of 1.52 ng/ml (**Table 3**). Based on these results, 17-hydroxypregnenolone and 11-deoxycorticosterone were considered the strongest indicators for the detection of cortisol-producing ACC among serum steroid metabolites.

Clinical correlation to steroid metabolites in ACC

To investigate whether serum or urinary 17-ketosteroids can be used additionally as prognostic factors in cortisol-producing ACC, we analyzed the correlation between serum or urinary 17-ketosteroids, and clinical parameters including tumor size, ENSAT classification, Weiss score, Ki67-index, and overall survival in ACC (**Table 4**). Serum levels of 11-deoxycortisol showed a significant positive correlation with ENSAT classification (R-value: 0.80, P-value: 0.03) (**Table 4** and **Fig. 3A**). Further, serum levels of testosterone showed a significant positive correlation with Ki67-index (R-value: 0.86, P-value: 0.03) (**Table 4** and **Fig. 3B**). Although there was a gender difference in testosterone, a correlation between testosterone and Ki67 was observed in both men and women, and it was considered to be on the same correlation line (**Fig. 3B**). There was no significant correlation between Weiss score, tumor size, or overall survival, and any steroid metabolites. Further, no significant correlation was found between urinary 17-ketosteroids and any of the clinical parameters.

Discussion

In the present study, all basal levels of steroid precursors and DHEAS were significantly increased in cortisol-producing ACC compared to CPA. The immunoassay was a successful tool for diagnosis, and we recommend both 17-hydroxypregnenolone (androgen precursor) and 11-deoxycorticosterone (mineralocorticoid precursor) for the diagnosis of cortisol-producing ACC. Only 17-ketosteroids were measured as urine steroid metabolites in our study, but none of the 17-ketosteroid fractions were sensitive to ACC diagnosis. Based on these findings, we would like to propose that measurement of serum steroid

metabolites, especially 11-deoxycortisol (glucocorticoid precursor) and testosterone, as a simple non-invasive method for predicting progression and prognosis in patients with cortisol-producing ACC.

This so-called heterogeneity of steroidogenesis observed is a reflection of immature and dedifferentiated cell features, which are the hallmark of ACC [11, 13, 15-17]. To our knowledge, no serum or urinary steroid metabolites associated with prognostic factors in ACC have been demonstrated to date, but several papers have reported these were useful for the diagnosis of ACC [11-19]. Consistent with our recommendation, Schweitzer et al. have reported that combination of androgen precursor (17-hydroxypregnenolone, progesterone, and DHEA), mineralocorticoid precursor (11-deoxycorticosterone), glucocorticoid precursor (11-deoxycortisol), and sex hormone (DHESA and estradiol) was useful for ACC diagnosis [13]. Taylor et al. also have recommended androgen precursor (17-hydroxypregnenolone) and glucocorticoid precursor (11-deoxycortisol) for diagnosis of ACC [12]. As for urinary steroid metabolites, 11-deoxycortisol urinary metabolite tetrahydro-11-deoxycortisol (glucocorticoid precursor) [15-18] as well as 17-hydroxypregnenolone urine metabolite pregnenetriol and pregnenolone urine metabolite pregnenediol (androgen precursor) [17, 18] were recommended. Although all papers have suggested that some steroid hormone precursors were useful in the diagnosis of ACC, the differences between the type of steroid precursors may be due to differences in cortisol-producing ability within ACC and adrenocortical adenoma. Accurate 24-hour collections are often not easy to obtain; and blood collections are more convenient to patients. LC-MS/MS has less cross-reactivity to other metabolites, but it requires more time for processing and measurement than immunoassay. For these reasons, we recommend the measurement of serum steroid metabolites by immunoassay for early accurate diagnosis and assessment of malignancy in cortisol-producing ACC.

The reasons for the correlations between 11-deoxycortisol and ENSAT classification, or testosterone and KI67-index were not elucidated in our study. In light of that, we would like to base our analysis on previous reports. Cortisol-producing ACC has been known to produce androgen the most simultaneously to cortisol [24-26]. It was suggested that cortisol-producing ACC that produce male sex hormone at the same time might have higher cell proliferation. Besides, since 11-deoxycortisol is converted to cortisol by Cytochrome P450 Family 11 Subfamily B Member 1 (CYP11B1), it has been considered that a relative deficiency of CYP11B1 may occur in ACC with poor progression. In fact, expression of CYP11B1 has been shown to be downregulated in some ACC [27].

There are cases where a confirmed diagnosis of ACC cannot be made, or surgery cannot be performed due to poor general condition or severe complications. In such cases, a measurement of serum steroid hormone metabolites would be helpful for predicting pathological characteristics or prognosis in cortisol-producing ACC. However, our study is limited to cortisol-producing adrenal tumors and is a retrospective study with a small number of ACC samples and limited urinary hormone measurements. Further large-scale prospective research will be required in the future.

In conclusion, a combination of measurement of serum steroid metabolites is a highly informative noninvasive method for both diagnosing, and predicting the clinicopathological features associated with disease prognosis of cortisol-producing ACC.

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