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Estrogen Secreting Adrenal Adenocarcinoma in an 18-Month-old Boy: Aromatase Activity, Protein Expression, mRNA and Utilization of Gonadal Type Promoter

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Abstract. We examined clinical, endocrinological and molecular biological aspects of an estrogen-secreting adrenal carcinoma in an 18-month-old male to clarify the pathogenesis of this condition. An 18-month-old boy was referred for evaluation of progressive bilateral gynecomastia and appearance of pubic hair. The patient had elevated plasma estradiol (349 pg/ml) and testosterone (260 ng/dl) levels that completely suppressed FSH and LH levels, and was subsequently diagnosed with an adrenal tumor on the right side. After removal of a 300-g adenocarcinoma, gynecomastia regressed and essentially normal hormone levels were restored. Aromatase activity in the tumor tissue determined by the ^{3}H-water method was 71.0–104.4 pmol/min/mg protein. High levels of aromatase protein and mRNA in the tumor tissue were also demonstrated, while neither aromatase activity nor protein was detected in normal adrenal glands. To investigate the regulation of aromatase expression in the adrenal carcinoma, we examined the usage of alternate promoters responsible for aromatase gene transcription. In the present case, the amounts of aromatase mRNA utilizing gonadal types of exon 1c (I.3) and 1d (II) were significantly higher than those that using other exon 1s. This result suggested that the utilization of a gonadal-type exon 1 might be involved in the overproduction of aromatase in estrogen-secreting adrenal carcinoma.

Key words: Aromatase, Estrogen, Adrenal, Carcinoma

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AROMATASE cytochrome P-450, also known as estrogen synthetase, is an essential enzyme in the synthesis of estrogens through the aromatization of androgens, and forms an enzymatic complex along with NADPH-P-450 reductase. This enzyme is present in gonadal tissues such as ovary [1] and placenta [2, 3], but also in extragonadal tissues such as brain [4] and adipose tissue [5, 6]. However, aromatase expression has not been observed in normal adrenal tissues. Excessive or inappropriate aromatase expression in adrenal carcinoma is associated with abnormally high circulating estrogen levels and/or increased local estrogen concentrations in adrenal tissues. Elevated estrogen levels, which is either systemically delivered or locally produced, promote the growth of hormone-responsive tissues.

The human aromatase gene contains 10 exons spanning at least 70 kilobases and is located on chromosome 15q21, which contains a cluster of P450 genes. Tissue-specific regulation of aromatase mRNA is achieved through the alternate splicing of exon 1 and part of exon 2, driven by at least five major promoter regions located within the 5′-untranslated region of the gene. Exon 1a (I.1) is uti-

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Table 1. Insulin and GnRH stimulation test

	0	30	60	90	120 (min)
ACTH (pg/ml)	89	453	178	288	888
Cortisol (μg/dl)	11.4	14.1	13.8	12.9	12.9
BS (mg/dl)	87	50	57	62	60
LH (mIU/ml)	<0.2	0.4	0.6	0.4	0.4
FSH (mIU/ml)	<0.2	0.7	0.7	0.6	0.6

lized mainly for aromatase mRNA expression in placenta, 1b (I.4) utilized mainly in skin fibroblasts and fetal liver, and both 1c (I.3) and 1d (II) are utilized in the ovary and prostate, respectively [7, 8]*. Therefore, tissue and developmentally regulated utilization of alternate exons 1 of aromatase gene allows for a complex regulatory mechanism for the tissue-specific expression of human aromatase.

Mild breast development occurs in 30–65% of boys during puberty, but is usually a self-limiting condition. However, the development of severe gynecomastia in prepubertal and adolescent boys and young men are very rare and may be the result of an estrogen-secreting carcinoma, or due to the recently reported aberrant P-450 aromatase gene transcription [9], or some other cause. Studies of uterine, ovarian, testicular and adrenal tumors of various types and grades of malignancy have suggested that alternative promoter-usage may be associated with the mechanisms that underlie neoplastic transformation. The clinical, endocrinological and molecular biological aspects of an estrogen-secreting adrenal carcinoma in an 18-month-old male are described.

Subject and Methods

Subject

An 18-month-old boy was referred for evaluation of progressive bilateral gynecomastia and appearance of pubic hair. He was the product of a normal pregnancy and delivered at term with a birth weight of 2826 g. His mother had neither received drugs nor experienced signs of virilization during gestation and had never employed oral contraceptives. Breast-feeding continued for 10 months. Pubarche started at 6 months of age and was followed by growth of breasts noticed at 18 months. Family history was unremarkable. Physical examination revealed symmetric gynecomastia at Tanner pubertal stage II (TS-II) with definite glandular tissue palpable, facial acne, pubic hair at TS-II and testicular volume of 2 ml bilaterally. Body weight and height were 14.5 kg (+3.5 S.D.) and 83 cm (+0.65 S.D.), respectively. Bone age was 3 years and blood pressure 96/70. Magnetic resonance imaging of the abdomen revealed a right adrenal mass (6×9×10 cm). Chest X-ray was not suggestive of pulmonary metastases.

Endocrine evaluation and treatment

The patient had elevated plasma estradiol (E_2) (349 pg/ml), testosterone (T) (260 ng/dl) and dehydroepiandrosterone sulfate (DHEAS) (29500 ng /ml), with undetectable gonadotropin levels. Insulin-stimulated levels of ACTH and cortisol, and GnRH-stimulated levels of gonadotrophins were evaluated (Table 1). Basal plasma ACTH level was slightly above the upper normal range, and this value markedly increased after insulin induced hypoglycemia (regular insulin 0.10 U/kg, iv), whereas plasma cortisol levels remained unchanged.

These results suggested that the patient's tumor-free adrenal function might be abnormal. Plasma renin activity (PRA) levels was high (14.0 ng/ml/hr) which might also suggest a subclinical adrenal insufficiency. Gonadotropin levels were completely

* The nomenclature of the various aromatase first exons and corresponding promoters is unsettled. In this manuscript, we use the codes established by Harada *et al.* [7]; exons 1a, 1b, 1c and 1d, which correspond to promoters PI. 1 (placenta), PI. 4 (adipose tissue), PI. 3 (adipose tissue), and PII (gonads) reported by Simpson *et al.* [8], respectively. The latter are indicated in parentheses.

suppressed after GnRH injection (100 μg/m^2, iv).

The patient underwent exploratory laparotomy with resection of the right adrenal tumor. The weight of the adrenal mass was 300 g. There was no evidence of intraabdominal, paraaortic node, or hepatic metastases. Plasma hormone levels before and after surgery are shown in Table 2. After resection of the tumor, plasma DHEAS, T and E_2 levels quickly fell and serum cholesterol level became normalized. The tumor was histologically an adenocarcinoma and mitotane therapy was initiated. Postoperatively, the patient was maintained on supportive glucocorticoid therapy, which was stopped 6 months later. Gynecomastia and pubic hair rapidly disappeared. Examinations at the present time have been normal.

Quantity of aromatase activity

Tumor tissue aromatase activity was determined by the tritiated (^{3}H)-water method as previously reported by Thompson and Siiteri with some modifications [10]. Details of the assay are reported by Watanabe *et al.* [11]. Human full-term placental microsomal fractions were included as positive controls.

Quantity of aromatase mRNA

Tumor samples were homogenized in 5 volumes of 5 M guanidine thiocyanate containing 5 mM sodium citrate and 0.5% sodium sarcosyl, and total RNA prepared as described by Chirgwin *et al.* [12]. Aromatase mRNA was assessed in these samples by RT-PCR as previously described [7, 13]. Internal standard RNA (0.01 attomole of human aromatase RNA containing a 21-base insertion) and total RNA (1 μg) were reverse transcribed at 42°C for 40 min and amplified by PCR for 26 cycles in the presence of a FAM-labeled primer. A FAM-labeled sense primer corresponding to exon 3 and an antisense primer corresponding to exon 5 were used as PCR primers to allow quantitative analysis of aromatase mRNA. Aliquots of the fluorescent PCR products were mixed with 3 μl GENESCAN-1000 ROX (Applied Biosystems, Foster City, CA), DNA size markers labeled with the fluorescent dye ROX, and analyzed fluorometrically with a Gene Scanner 362. FAM-labeled PCR products showed two peaks corresponding to amplified products of aromatase mRNA and the internal standard RNA at positions approximately 378 and 399 bp, respectively. The amount of aromatase mRNA in each sample was calculated from the peak areas of fluorescent products by the internal standard method as previously described [7]. No fluorescent PCR products were detected in the absence of RNA.

Western blot analysis

Tissue samples were homogenized in 10 volumes ice-cold 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.5) and 1 mM ethylenediamine tetraacetate us-

Table 2. Plasma hormone levels of a boy with adrenal cancer and gynecomastia

	Before surgery	After surgery	Normal range
E_2 (pg/ml)	349	<10	<10
T (ng/dl)	260	2	3–13
DHEAS (ng/ml)	29500	23	59–87
ACTH (pg/ml)	89 (888*1)	7.9	4.8–50.8
Cortisol (ug/dl)	11.4	22.1	3.0–21.0
Aldosterone (pg/ml)	135	55.9	66.4–217.6
LH (mIU/ml)	<0.2 (0.6*2)	0.31	0.04–0.77
FSH (mIU/ml)	<0.2 (0.7*2)	1.08	0.18–2.06
17-OHP (ng/ml)	0.6		
T-cho. (mg/dl)	16	163	115–220

*1; insulin tolerance test, basal (max)
*2; LH-RH test, basal (max)

ing a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Microsomal fractions were prepared by successive centrifugation from the homogenates as previously described [14]. Final microsomal pellets were suspended in a minimum volume of 50 mM Tris-HCl (pH 7.5) containing 20% glycerol and 1 mM ethylenediamine tetraacetate. Protein content was estimated using the BCA protein assay (Pierce Chemical Co., Rockford, IL), with BSA as a standard. SDS-PAGE was performed according to the procedure of Laemmli [15]. Microsomal proteins were loaded on 9% SDS polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes, immunoreacted with rabbit polyclonal antibody [16, 17] raised against human placental aromatase and visualized with alkaline phosphatase-conjugated goat antirabbit IgG (Bio-Rad Laboratories, Richmond, CA).

Analysis of alternative exon 1 usage in aromatase mRNA

The utilization of alternative aromatase gene exon 1 usage was determined by RT-PCR using sense primers specific for exons 1a (I.1), 1b (I.4), 1c (I.3) and 1d (II) and a fluorescent dye-labeled antisense exon 3-specific primer as described previously [7, 13, 18]. Harada *et al.* previously reported that exon 1a (I.1) was utilized in human placenta, 1b (I.4) in skin fibroblasts and fetal liver, 1c (I.3) in ovary and 1d (II) in prostate. Fluorescent PCR products were analyzed with a 362 Gene Scanner (Perkin-Elmer Corp.). Aromatase mRNAs transcribed from exons 1c (I.3) and 1d (II) yielded PCR products corresponding to 357 and 371 bp, respectively. Genescan-1000 ROX was used as an internal size standard as described above.

Results

Aromatase activity in the tumor

Very high levels of aromatase activity were observed in microsomes obtained from tumor tissue (71.0–104.4 pmol/min/mg protein), which were not detected in normal adrenal tissues.

Aromatase mRNA in the tumor

Aromatase mRNA levels in the tumor (18.0–21.4 attomoles (amol)/μg RNA) were significantly increased compared to undetectable levels in normal adrenal tissues.

Aromatase protein expression in the tumor

To demonstrate whether tumor aromatase expression levels reflected protein levels, Western blotting analysis of aromatase in adrenal cancer and human placenta tissues was performed. Fig. 1 shows an immunoblot of the tumor and human placenta samples with anti-human aromatase antibody. Microsomes from both tumor and human placenta samples gave single immunoreactive protein bands corresponding to 51,000 dalton molecular weight, which was consistent with the molecular weight of purified human aromatase [19].

Preferential usage of multiple exons 1 in tumor aromatase mRNA

RT-PCR was used to determine which types of

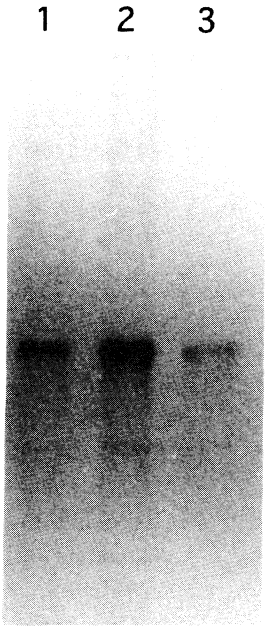

Fig. 1. Western blot analysis of aromatase. Lane 1, adrenal microsomes from the adrenal tumor; Lane 2, microsomes from healthy control placenta; Lane 3, purified aromatase. In normal adrenal glands, aromatase was not deteced (data not shown).

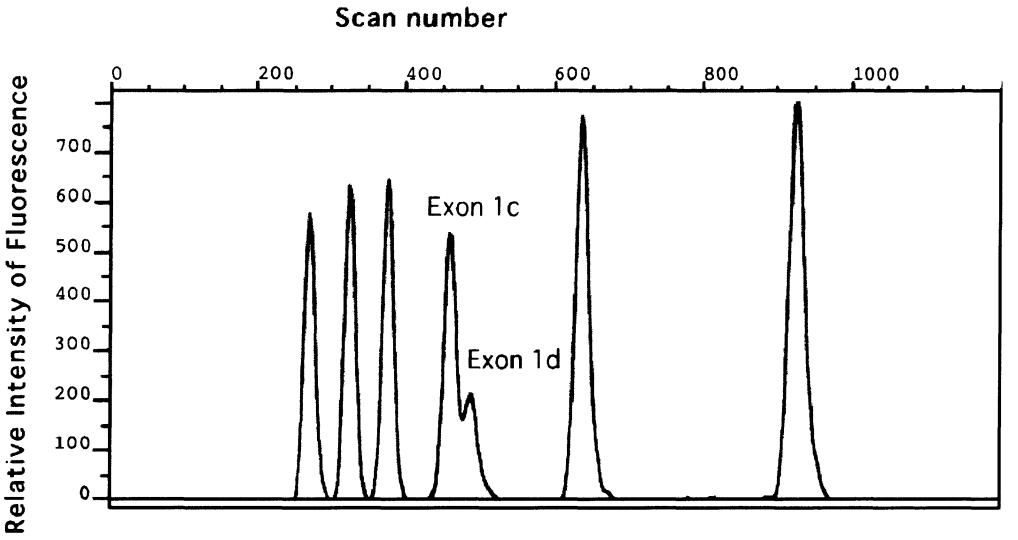

Fig. 2. RT-PCR analysis showing utilization of alternate exon 1 sequences of the human aromatase gene in an adrenal tumor. The GENESCAN-1000 ROX internal size standards produced five peaks, corresponding to 263, 291, 318, 439 and 557 bp.

multiple exons 1 and promoters were used in the tumor. Relative preference of exons 1 utilization was assessed fluorometrically as shown in Fig. 2. Exon 1c (I.3) and 1d (II) were exclusively used in the tumor aromatase mRNA.

Discussion

This report presents a feminizing adrenocortical carcinoma in an 18-month-old boy with gynecomastia and virilization. As the patient had highly elevated E_2 levels, we examined aromatase activity, aromatase protein, and tumor-specific aromatase gene promoter usage. To our knowledge, the patient was the youngest reported with an estrogen producing adrenal tumor [20–35], and constituted the second report describing the preferential usage of alternative exons 1 of aromatase gene in adrenal tumors (Table 3).

The patient had a relatively high basal ACTH and PRA, an exaggerated ACTH response but no cortisol response to insulin induced hypoglycemia. This indicated that the patient's normal adrenal gland did not secrete cortisol and aldosterone normally. An extremely low cholesterol level in this patient was apparently due to the uptake of cholesterol by the 300 g adrenal tumor as an initial substrate of steroid biosynthesis. This is supported by the observation that the low cholesterol level before surgery returned to normal after excision of the tumor. Since 80% of adrenal steroidgenesis is accounted for by the uptake of plasma cholesterol, the patient's extremely low cholesterol level, due to the excessive uptake of cholesterol by the adrenal tumor, may have been the cause of the adrenocortical insufficiency in the normal adrenal gland. This is the fourth reported case of an association between hypocholesterolemia and adrenal tumors, and the first accompanied by an estrogen secreting tumor (Table 3).

DHEA, the major adrenal androgen, is converted to androstendione and then testosterone, both of which are substrates for aromatase, which produces estrogen. However, it has not been directly demonstrated that estrogens are synthesized by normal adrenal glands. In this patient, circulating E_2 levels were 300–400 times higher than in normal subjects.

The patient had higher DHEA-S levels than those observed in previously reported estrogen-producing adrenal tumors. As the high levels of estrogens and testosterone, and the low levels of gonadotoropins promptly returned to normal after removal of the carcinoma, we concluded that the massive amounts of E_2 and testosterone were secreted directly by the adrenal tumor. This hypothesis was supported by the demonstration of aberrant aromatase expression in the adrenal tumor. The tumor-expressed aromatase had a very high level of activity at 71.0–

Table 3. Summary of reported cases of estrogen secreting adrenal tumor

Sex/Age	A	P	E2	DHEAS	Clinical presentation	Ref)	
M/48 y.			367	970	gynecomastia	Boyar	(20)
M/27 y.			250	1810	gynecomastia	Boyer	(20)
M/43 y.			132	7800	recurrent carcinoma	Nogeire	(21)
F/21 m.			170	14080	breast enlargement	Wohltmann	(22)
M/37 y.			300		gynecomastia	Nishiki	(23)
F/ 6 y.			330	3100	breast enlargement	Drop	(24)
M/ 6 y.			40–56	3100	gynecomastia	Itami	(25)
M/48 y.			72	1505	gynecomastia	Seta	(26)
F/29 y.			105	6600	amenorrhea/hirsutism	Ho Yuen	(27)
M/48 y.			7.9		gynecomastia	Mersey	(28)
M/58 y.			208		gynecomastia	Saadi	(29)
F/ 6 y.			30	5233	virilization	McKenna	(30)
M/33 y.			62	3727	Cushing/gynecomastia	McKenna	(30)
F/63 y.			300	2630	Cushing/vaginal bleeding	Singer	(31)
M/45 y.			69	18300	gynecomastia	Zayed	(32)
M/19 y.	3.6		1910	4.7 (DHEA)	gynecomastia	Kimura	(33)
F/65 y.	2.0		81	w.n.l	vaginal bleeding	Goto	(34)
M/29 y.	4.2	G	515	463	gynecomastia	Young	(35)
M/18 m.	71–104.4	G	349	29500	gynecomastia	Watanabe	

A; aromatase activity (pmol/min/mg protein), P; promoter type, G; gonadal type, w.n.l; within normal limit, E2; estradiol (pg/ml), DHEAS; dehydroepiandrosterone sulfate (ng/ml)

104.4 pmol/min/mg protein, equivalent to that found in full-term human placenta, whereas no aromatase activity was detected in normal adrenals. The high aromatase levels in the tumor tissue were also examined by investigating aromatase protein and mRNA levels, which were undetectable in normal adrenal glands.

We demonstrated that the aromatase gene in the adrenal tumor tissue utilized the gonadal type exons 1c (I.3) and 1d (II). Tissue-specific expression of aromatase is regulated through alternative use of multiple exons 1 and promoter [7, 36]. In fact, each promoter region has been shown to contain binding sites for tissue-specific transcription-regulatory factors [37]. Recently, a switching of the alternative exons 1 was found under pathological conditions [38, 39]. A switching from exon 1b to exons 1c or 1d was often observed in association with elevated expression of aromatase in breast cancer tissues [40]. Similar observation was also reported in the tumors of endometrium, ovary, liver, and colon [11, 13, 41]. As reported in breast cancer tissues [42], recruitment of tissue-specific transcription-regulatory factors (1bSEBP) to binding sites (1bSE) in the promoter of exon 1b seems to be interfered with when various factors (i.e. PGE2)/cytokines (i.e. IGF-I, IGF-II, IL-1, and IL-6) were secreted from host and tumor cells [43], and consequently, a switching would occur. The expression of aromatase in the present case of adrenal adenocarcinoma might be caused by a similar molecular mechanism.

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