FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 42, No. 4, 2004
pp. 215-220

Immunoexpression of androgen receptors and aromatase in testes of patient with Klinefelter's syndrome

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Abstract: Klinefelter's syndrome (47, XXY) is the most common chromosome aneuploidy in men and is usually characterized by underdeveloped testes and sterility. The aim of the present study was to detect cellular distribution of androgen receptors (AR) and aromatase in testes of patient with KS. The tissue sections were processed for morphological and immunohistochemical staining. Additionally, levels of FSH, LH, PRL, estradiol, and testosterone were measured in the plasma. Morphological analysis revealed a complete absence of spermatogenesis. No germ cells were present in seminiferous tubules. In some tubules, nests of apparently degenerating Sertoli cells were found. In the interstitium, Leydig cell hyperplasia was observed. Using immunohistochemistry, nuclear AR staining was detected in Sertoli cells and peritubular cells, whereas in Leydig cells the staining was exclusively cytoplasmic. The immunostaining of aromatase was detected in the cytoplasm of Sertoli cells and Leydig cells. Increased levels of gonadotropins and decreased level of testosterone concomitantly with the cytoplasmic localization of AR in Leydig cells might contribute to the impaired testicular function in patient with KS.

Key words: Androgen receptor - Aromatase - Testis - Klinefelter's syndrome - Immunohistochemistry

Introduction

Klinefelter's syndrome (KS) was described for the first time in 1942 [18]. It is characterized by abnormal number of X chromosomes and primary gonadal insufficiency. In its classic form, there are varying degrees of eunuchoidism, gynecomastia and undersized, firm testes [22]. Histologically, the testes show a progressive failure of spermatogenesis, accompanied by tubular sclerosis and Leydig cell hyperplasia. Recently, Lue *et al.* [21] have established an experimental animal model for KS (XXY male mice), in which depletion of germ cells is associated with an increase in levels of serum gonadotropins as it is in patients with KS.

There are several reports showing functional alterations in testes of KS patients. β-endorphin expression has been detected, whereas no positive staining has been found in normal human testes [31]. Recently, serum inhibin B, a marker for spermatogenesis was measured in patients with KS as well as in controls. During late puberty its levels decreased gradually to unmeasurable amounts in adult patients with KS, while remaining unchanged in the controls [12]. By means of immunohistochemistry, Yamamoto *et al.* [37] has shown reduced functional Leydig cell activity in KS testes in comparison with normal human testes. No data is available, however, on immunolocalization of androgen receptors (AR) in patients with KS.

It is well known that both androgens that regulate initiation and maintenance of spermatogenesis and their receptors are required for normal germ cell development [1]. The androgen action is mediated by a single AR which is almost exlusively nuclear and upon ligand binding undergoes a conformational change that involves displacement of heat shock proteins from the ligand-binding domain. Then, the resulting active form of the AR is capable of dimerization, translocation to the cell nucleus and binding to specific DNA sequence, thus regulating the expression of androgen-responsive genes [38].

Genetic defects associated with male infertility include microdeletions of spermatogenic genes on the Y-chromosome and/or genetic disorders affecting the secretion and action of gonadotropins leading to androgen deficiency [11, 17, 38]. In a recent study, Suzuki

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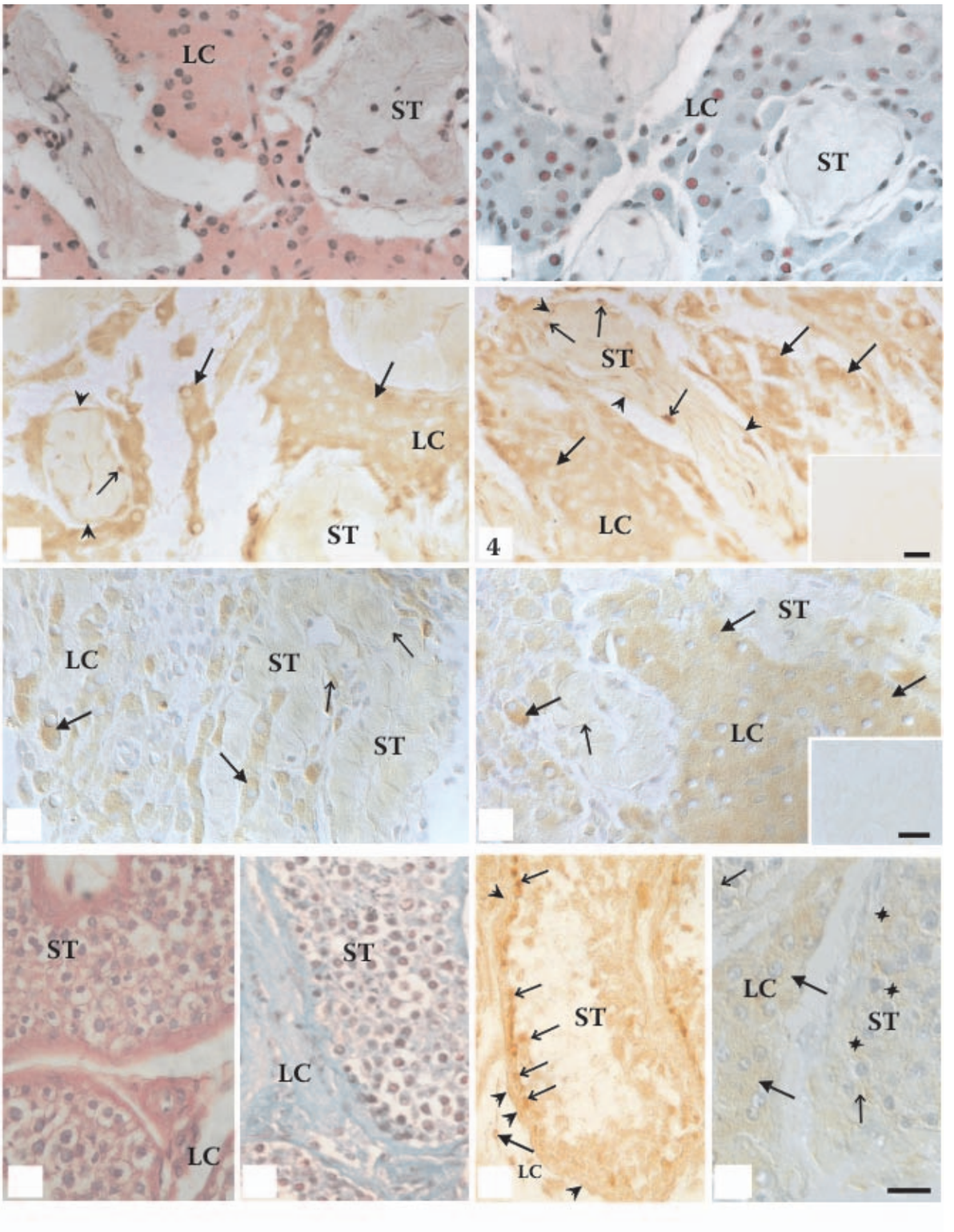

Figs 1-10. Morphology (**Figs 1, 2, 7, 8**). AR-immunodetection (**Figs 3, 4, 9**), and aromatase immunodetection (**Figs 5, 6, 10**) in testis of patient with KS and of fertile graft donor. ST - seminiferous tubules, LC - Leydig cells. Scale bars = 20 μm. **Figs 1, 2.** H+E staining (**Fig. 1**) and Masson-Goldner staining (**Fig. 2**) of testicular tissue of patient with KS. Note hyalinization of seminiferous tubules and absence of germ cells. In the interstitial tissue, hypertrophy and hyperplasia of Leydig cells can be seen. **Figs 3, 4.** Immunolocalization of AR in KS patient.

et al. [34] have suggested that *AR* gene abnormality does not constitute an important factor for impaired spermatogenesis in patients with KS. It is in agreement with results of Sasagawa *et al.* [29] who have detected no mutations in any of the *AR* gene exons 1-8 in males with cryptorchidism, hypospadias, micropenis, or impaired spermatogenesis. These facts indicate that an alteration of the *AR* gene is rare in these males. It is therefore likely that besides chromosomal abnormality, a main cause of KS, elevated levels of LH and FSH with concomitant low level of testosterone are responsible for further degenerative alterations of human testes.

Recently, it has been established that estrogens also play an important role in the regulation of spermatogenesis and maintenance of male fertility [8, 9, 15, 16]. The biosynthesis of estrogens from androgens is catalyzed by the microsomal enzyme, P450 aromatase (P450arom) [10, 13]. Therefore, it is worth investigating, whether biosynthesis of testosterone and its action as well as testosterone metabolism is altered in patients with KS. The aim of this study was to detect cellular distribution of AR and aromatase in testes of patient with KS and to compare their localizations with that in human testes obtained from fertile graft donors. Additionally, to know more about the hormonal profile of the patient, levels of FSH, LH, PRL, estradiol, and testosterone were measured in the plasma.

Materials and methods

Material. Testicular biopsy was obtained from 31-year-old man with KS diagnosed for ICSI-PESA/TESA procedures (ICSI - intracytoplasmic sperm injection; PESA - epididymal sperm aspiration; TESA - testicular sperm aspiration). Since testicular alterations are conjugated in both testes, a biopsy only from one testis was performed. Human testes of fertile men, graft donors (victims of a sudden death), served as the control. For the procedure approval was obtained from the institutional ethical committee.

The specimens were fixed in 4% formaldehyde freshly prepared from paraformaldehyde in phosphate buffered saline, embedded in paraplast (Monoject Scientific Division of Scherwood Medical, St Louis MO, USA), and processed for routine histology. Morphological analysis was carried out after haematoxylin-eosin (H+E) or Masson-Goldner trichrome (acid fuchsin, Ponceau 2R, Orange G, and light green) stainings.

Immunohistochemistry. Immunohistochemistry was performed on 6-μm-thick sections which were mounted on slides coated with 3-aminopropyl-triethoxysilane (APES; Sigma, St Louis MO, USA), deparaffinized and rehydrated. To optimize immunostaining, sections were immersed in 10 mM citrate buffer (pH 6.0) and treated in a microwave oven (2 × 5 min, 600 W) for antigen unmasking [4]. Nonspecific staining was blocked twice, with H_2O_2, for 7 min to inhibit endogenous peroxidase activity, and with normal horse or goat serum respectively, for 15 min, to block nonspecific binding sites. After that, sections were processed for visualization of either AR or aromatase using the immunohistochemical technique [3, 20]. In short, sections were incubated overnight at 4˚C in a humidified chamber in the presence of primary antibodies: (1) for the AR, a rabbit polyclonal antibody (1:10; Novocastra Lab., Newcastle upon Tyne, UK) and (2) for aromatase, a mouse anti-human cytochrome P450 aromatase (1:10; Serotec Ltd, Oxford, UK). Next, biotinylated secondary antibodies, goat anti-rabbit IgG (1:400; Vector Lab, Inc., Burlingame, CA, USA) and horse anti-mouse IgG (1:400; Vector Lab., USA) were applied, respectively . Finally, avidin-biotinylated horseradish peroxidase complex (ABC/HRP; 1:100; Dako, Glostrup, Denmark) was used. After each step of the above procedure, the sections were carefully rinsed with Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl , pH 7.6); the antibodies were also diluted in TBS. The antibodies bound to the sections were visualized by 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, MO, USA) in TBS containing 0.01% H_2O_2 and 0.07% imidazole. Some of the sections were counterstained with Mayer's haematoxylin. Respective controls were processed using identical protocols, except for the primary antibodies which were omitted and substituted by nonimmune IgGs. Each immunostaining procedure was repeated on 3-4 sections and was always accompanied by control reaction. The sections were examined for the presence and intensity of immunostaining. Aromatase and AR immunostainings were designated as weak, moderate or strong on the basis of visual examination of cytoplasmic or nuclear localization of the antigen. Cells without any specific immunostaining were considered as negative.

Hormone measurements. For measurements of follitropin (FSH), lutropin (LH), prolactin (PRL), estradiol (E2), testosterone (T), and dihydrotestosterone (DHT), the blood samples were collected between 8.00 and 10.00 a.m. from brachial vein. Then, plasma was separated for subsequent hormone assays. Steroid hormone levels were measured enzymatically with a chemilluminescent marker using the automatic Bayer's apparatus (ACS:180^{TM}SE; Bayer Co.).

Results

Morphology

Morphological analysis revealed a complete cessation of spermatogenesis in patient with KS. The seminiferous tubules showed different degrees of sclerosis, from thickening of the basement membrane to complete hyalinization. They were lined by Sertoli cells only. In some tubules, however, nests of apparently degenerating Sertoli cells were found. Germ cells were absent. In the interstitial area, large clusters of Leydig cells were observed (Figs 1, 2). Morphological alterations did not appear in the testes of fertile organ donors (control) where normal spermatogenesis was observed (Figs 7, 8).

Note a strong immunostaining for AR in the cytoplasm of Leydig cells (arrows) and in the nuclei of Sertoli cells (open arrows) and peritubular cells (arrowheads). **Figs 5, 6.** Immunolocalization of aromatase in KS patient. Moderate to strong immunostaining in the cytoplasm of Leydig cells (arrows) and Sertoli cells (open arrows) is visible. Counterstained with Mayer's hematoxylin. **Figs 7, 8.** H+E staining (**Fig. 7**) and Masson-Goldner staining (**Fig. 8**) of testicular tissue from fertile graft donor. Normal spermatogenesis can be observed in seminiferous tubules. **Fig. 9.** Immunolocalization of AR in fertile graft donor. Moderate nuclear AR immunostaining in Leydig cells (arrows), very strong nuclear AR immunostaining in Sertoli cells (open arrows) and weak nuclear AR immunostaining in peritubular cells (arrowheads) can be seen. **Fig. 10.** Immunolocalization of aromatase in fertile graft donor showing strong immunostaining in the cytoplasm of Leydig cells (arrows), and moderate immunostaining in both, Sertoli cells (open arrows) and germ cells (asterisks).

Immunohistochemistry

Using immunohistochemistry, in the testis of patient with KS, nuclear AR staining was detected in Sertoli and peritubular cells, whereas in Leydig cells the immunostaining was exclusively cytoplasmic (Figs 3, 4). In control testis, only nuclear localization of AR was detected in Sertoli, Leydig, and peritubular cells (Fig. 9). Additionally, the nuclei of smooth muscle cells in the walls of blood vessels were strongly immunostained in control testes, while smooth muscle cells of KS patient were immunonegative for AR.

In the testis of patient with KS, cytoplasmic aromatase immunostaining was observed in Sertoli cells and Leydig cells (Figs 5, 6), while in control testis a weak aromatase immunoreactivity was also present in germ cells (Fig. 10). In control sections, no immunostaining for AR or aromatase was found when the incubation was performed without the primary antibodies, respectively (Figs 4 and 6, insets).

Levels of hypophyseal and gonadal hormones in plasma of patient with KS

The levels of LH and FSH were very high when compared with the reference range for men aged between 20 and 70 yrs (Table 1). The levels of other hormones did not exceed the normal range with the exception of bioavailable testosterone which was lower .

Table 1. Plasma hormone levels in patient with Klinefelter's syndrome

Hormone	Unit	Result	Reference range for men aged between 20 and 70 yrs
LH	mIU/ml	22.0	1.5-9.3
FSH	mIU/ml	53.4	1.4-18.1
PRL	ng/ml	11.4	2.1-17.1
E_2	pg/ml	18.6	10-52
T	ng/ml	6.2	2.4-8.2
T (bioavailable)	ng/ml	0.34	>0.7
DHEA-S	µg/dl	312.0	80-560

Discussion

Our study shows that androgen receptors and aromatase are present in testicular cells of patient with KS, however, the cytoplasmic staining of AR in Leydig cells indicates the existence of functionally inactive AR, possibly due to a very low level of bioavailable, free testosterone. The latter is likely associated with the elevated levels of LH and FSH, as measured in the present study. Low level of serum testosterone and elevated gonadotropins with concomitant degeneration of seminiferous tubules have also been described by Ogawa and Yoshida [25]. In the absence of androgens, AR is known to be located in the cytoplasm [14]. It is well known that there are two forms of steroid-hormone receptors: the unliganded receptor in the cytoplasm and hormone-receptor complex in the nucleus. A nuclear translocation mechanism was hypothesized as a necessary link between the two forms [14]. This is supported by the studies of Truss and Beato [35] who have found AR present in the cytoplasm without hormonal stimulation. The cytoplasmic distribution of AR may reflect receptor localization in the lysosomal compartment possibly involved in receptor degradation.

According to Mor *et al*. [22] the interstitial cells in the gonad of patient with KS exhibit varying degrees of functional abnormality. The authors described hyperplastic Leydig cells that represented a true hyperplasia, not a result of condensation due to a decrease in the volume of the testis after tubular sclerosis. Also Regadera *et al.* [27] found a high percentage of multivacuolated and pleomorphic Leydig cells in testes of KS patients, whereas in normal testes only typical Leydig cells with very low percentage of multivacuolated forms were noticed. In our study, in the testis of KS patient we also observed hyperplasia of Leydig cells in almost all sections examined. These cells, frequently multivacuolated, were visible among abundant connective tissue as stained by a Masson-Goldner trichrome. In the opinion of Mor *et al*. [22], impaired hormonal function of the testes in KS may be also related to the absence of annulate lamellae in Sertoli cells, and to abnormal mitochondria, absence of Reinke crystals, and the presence of other microcrystals in Leydig cells. The ultrastructural features of Leydig cells support the hypothesis that the alterations in cellular steroidogenesis are associated with Leydig cell morphology. Moreover, Martin *et al.* [23] have suggested that the thickening and invaginations of the basal lamina in seminiferous tubules with initial sclerosis in KS might be related to alterations in either Sertoli cells or in the inner layer of peritubular cells which acquire a fibroblastic pattern.

As shown herein by immunohistochemistry, in contrast to Leydig cells, both Sertoli and peritubular cells exhibit a nuclear AR staining, what indicates a direct effect of androgen action on both types of the cells. However, smooth muscle cells surrounding the walls of blood vessels were immunonegative for AR. On the contrary, in the control testis, smooth muscle cells expressed nuclear AR staining, as did all somatic cells. The latter results are in agreement with data of Bergh and Damber [2] and Suarez-Quian *et al.* [33] who, besides somatic cells of rat and human testes, have found smooth muscle cell layer of testicular blood vessels to be

equipped with AR. It seems likely that in testes of KS patient, smooth muscle cells are not a direct target for androgens since they do not possess AR. No immunoexpression of AR has also been found in cryptorchid mouse and human testes [19, 28] indicating the impaired androgen action in testes with severe disruption of spermatogenesis.

In the present study, we also localized aromatase in both, Leydig and Sertoli cells, however, the intensity of immunostaining was not the same, being apparently stronger in Leydig cells of KS patient than in those of the control testis. It should be stressed that the monoclonal antibody against aromatase used in our study is now highly recommended for mammalian including human tissues [36]. Turner *et al.* [36] and Carpino *et al.* [7] using anti-human monoclonal P450arom IgG as the primary antibody have shown for the first time in human testes that Leydig cells, Sertoli cells, germ cells, and epithelial cells of ductuli efferentes as well as those of proximal caput epididymis express aromatase. These findings are of special importance confirming through ubiquitous distribution of aromatase, an important role of estrogens in the regulation of spermatogenesis in humans.

The stronger aromatase immunostaining in KS patient detected in our study indicates the higher rate of androgen conversion to estrogens and, in consequence, may lead to the imbalance of steroid hormones. According to Sharpe [30], the androgen/estrogen ratio is essential for normal function of the testes. The widespread distribution patterns of aromatase in several tissues corroborate Sharpe's hypothesis that the balance between those steroid hormones is crucial for many androgen/estrogen target sites [for review see 10, 13, 15]. Therefore, low level of bioavailable testosterone and relatively high estradiol level as found in the present study may be responsible for further morphological and functional alterations in testes of KS patient. Intensely immunostained Sertoli cells as well as high aromatase activity have also been observed in the testicular tumors of patients with Peutz-Jeghers syndrome [5, 6]. These results are also in agreement with our own findings [32] and suggest that Sertoli cells may express aromatase under abnormal conditions. In another study, Nakazumi *et al.* [24] have found that impaired spermatogenesis in patients with testicular germ cell tumor is caused by increased tumor size and/or by increased aromatization and *in situ* estrogen production in Leydig cells. Recently, in a clinical study of infertile men with KS, Raman and Schlegel [26] have shown that testosterone/estradiol ratio can be improved during treatment with an aromatase inhibitor, testolactone, leading to favorable changes in semen parameters.

Taking into account data reported herein, it may be concluded that KS includes hormonal imbalance between gonadotropins and steroid hormones leading to deficiency of bioavailable testosterone and altered AR and aromatase expression.

Acknowlegments: This work was supported by the State Committee for Scientific Research, grant BW/IZ/5b/2004 to M. K-B.

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Accepted July 16, 2004