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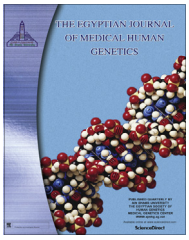

ORIGINAL ARTICLE

Partial association of restriction polymorphism of the ligand binding domain of human androgen receptor in prostate cancer

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Abstract *Background:* Human androgen receptor (AR) functions as a steroid-hormone activated transcription factor. The receptor binds to its ligand (testosterone or dihydrotestosterone) and is translocated to the nucleus to stimulate the transcription of androgen responsive genes. Mutations in the ligand binding domain (LBD) impair the receptor activity and play a crucial role in the development and progression of prostate cancer (PCa).

Materials and methods: This work was designated to investigate the restriction integrity of the LBD and its association with benign prostatic hyperplasia (BPH) and prostate cancer. Exons of this domain (exons: 4–8) were amplified from prostate tissue of BPH and PCa patients and the restriction polymorphism was investigated by *SmlI*, *HphI* and *Tsp45I* restriction enzymes in both BPH and PCa groups.

Results: Data revealed the integrity of exons 4–6 in both BPH and PCa patients. Exons 7 and 8, however have kept their constitutional pattern only in BPH patients. *Hph1* site showed an abnormal restriction pattern in 40% and 26.7% of PCa patients. Also, *Tsp45I* demonstrated restriction polymorphism in 20% and 13% of PCa patients.

Conclusion: Our results indicate that the loss of the restriction integrity in the C-terminal part (exons: 7 and 8) of the LBD is associated with the progression of benign prostatic hyperplasia to prostate cancer.

1. Introduction

Prostate cancer is the sixth leading cause of cancer-related deaths among men [1]. Prostate cancer cells usually acquire a large number of genetic alterations including point mutations, deletions, amplifications and translocations. Also, a wide range of epigenetic modifications was reported, such as

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Table 1 Mean ages and prostate specific antigen (PSA) in benign prostatic hyperplasia and prostate cancer patients.

	Groups			
	Benign prostatic hyperplasia		Prostate cancer	
n	15		15	
	Age (yr)	PSA (ng/ml)	Age (yr)	PSA (ng/ml)
Mean	58.9	7.1	61.9	1.9[a]
SEM	2.59	1.7	2.9	0.4
Range				
Minimum	48.0	1.40	47.0	0.5
Maximum	82.0	21.2	75.0	1.8
Normality test (*KS*)	0.135	0.21	0.218	0.312
P value	> 0.1	> 0.1	> 0.1	> 0.1

[a] Significant difference between the indicated group vs BPH.

changes in the acetylation and/or methylation patterns in addition to chromatin remodeling [2,3]. This type of cancer develops through a gradual progression through which the benign prostatic epithelial cells progress to prostatic intraepithelial neoplasia (PIN), invasive adenocarcinoma, distant metastatic disease and androgen refractory metastatic disease [4]. This transformation is characterized by several diagnostic nuclear morphological features, such as nuclear and nucleolar enlargement and alterations in chromatin structure [5].

The dependence of prostate cancer on androgen stimulation was described few decades ago [6]. Androgens (testosterone or dihydrotestosterone) bind to the androgen receptors (AR) and the liganded receptor is translocated to the nucleus, binds the androgen responsive elements (ARE), affects the transcription of androgen-regulated genes (e.g. prostate-specific antigen, PSA) and ultimately stimulates the proliferation and inhibits apoptosis of prostate cancer cells.

Many studies have reported that androgen receptor mutations and polymorphism are deeply involved in prostate cancer [7] and [8]. This receptor is encoded by AR gene, located on the X chromosome, at Xq11-12 [9], which consists of 6 functional domains "labeled A through F" [10]. The ligand binding domain (LBD) (domain E) represents the attachment site of androgens and has activation function-2 (AF2), which is responsible for agonist induced activity. Also, the LBD functions as a nuclear export signal [11]. Mutations in LBD were found to restrict the binding of testosterone or DHT to the receptor and subsequently impair AR mediated transactivation. The literature has reported unlimited number of genetic abnormalities in different domains of the AR gene. Most of these genetic abnormalities were detected in prostate cancer (PCa) patients. Also, the majority (79%) of mutations, identified in the LBD in PCa patients, were clustered to three discrete regions that influence the receptor activity [12].

A few studies have investigated the genetic integrity of AR gene during the progression toward cancer, especially in patients with androgen insensitivity syndrome (AIS) and/or patients with prostate cancer. Hence, this work was designated to investigate the genetic integrity of LBD in patients with benign prostatic hyperplasia (BPH) and those who developed prostate cancer (PCa) compared to the constitutional restriction pattern.

2. Subjects and methods

2.1. Patient population and grouping

The study included 30 male patients. All subjects were selected from inpatient of the catheterization units of El-Hussein hospital, Al-Azhar University, and Shebin El-Kome hospital and from El-Mataria Institute. Samples were obtained after surgery under informed consent, following the regulations and approval of the ethics committee and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. Patient's medical history and PSA levels were recorded with special attention to any associated medical problems. The inclusion criteria were benign prostatic hyperplasia (BPH) or prostate cancer (PCa). Patients were divided into two groups. Group I ($n = 15$) included patients with BPH, their ages ranged from 48 to 82 years (average ± SEM: 58.9 ± 2.59 years), whereas group II included 15 patients diagnosed with PCa, their ages ranged from 47 to 75 years (average ± slandered deviation: 61.8 ± 2.9 years) (Table 1).

2.2. Extraction of genomic DNA and exons amplification

Genomic DNA was extracted from tissues of BPH and PCa tumors using QlAmp DNA (GENELUTE) following the manufacturer's instruction. The DNA yield was measured at 260 nm and its integrity was tested by running onto 1.5% agarose gel. Exons 4–8 of the LBD were amplified using segment specific primer pairs (Table 2). In each amplification, 100 ng/μl was amplified in a PCR mix containing 1X (5 μl of 10X buffer) (Stratagene), 0.2 mM each dNTP (2 μl dNTPs mix), 100 pmol/μl (2 μl) of each exon specific primers (Integrated DNA Technologies, USA) and 2.5 U (0.5 μl) *Taq* DNA polymerase (GENE KRAFT, Germany). The reaction mixture was brought to 50 μl with molecular biology grade water.

Reactions of exons 4/5 and 6 were subjected to a thermal cycling program consisting of initial denaturation at 94 °C for 5 min, followed by 35 cycles of annealing at 55 °C for 30 s, primer extension at 72 °C for 40 s and denaturation at 94 °C for 30 s followed by a single extension at 72 °C for 5 min. For

Table 2 Sequence of primers used to amplify different LBD exons.

Exon number	Primer	Direction	Nucleotide sequences	Band size (bp)
4/5	LBD4/5s	Sense	5′-gtgattttcttagctagggc-3′	424
	LBD4/5as	Antisense	3′-atccccctatctcatgctc-5′	
6	LBD6s	Sense	5′-tggtaaacttcccctcattc-3′	249
	LBD6as	Antisense	3′-taatggcaaaagtggtcctc-5′	
7	LBD7s	Sense	5′-tgtggtcagaaaacttggtg-3′	299
	LBD7as	Antisense	3′-ctctatcaggctgttctccc-5′	
8	LBD8s	Sense	5′-gccacctccttgtcaaccct-3′	288
	LBD8as	Antisense	3′-agaggagtagtgcagagtta-5′	

exons 7 and 8 amplification, reactions were subjected to a thermal cycling program consisting of initial denaturation step at 94 °C for 5 min, followed by 35 cycles of annealing at 58 °C for 40 s, primer extension at 72 °C for 1 min and denaturation at 94 °C for 30 s followed by a single extension step at 72 °C for 10 min. At the end of the reaction, the amplification products (10 µl) resolved onto 2% agarose gel containing ethidium bromide for 30 min at 70 V, where DNA bands were visualized and photographed under a UV transilluminator.

2.3. Restriction analysis

Restriction analysis polymorphism of the LBD was undertaken using *SmlI*, *HphI* and *Tsp45I* (FERMINTASE, Germany). Each reaction included 1X of enzyme buffer, 5 µl of the targeted PCR product and 10 U of the restriction enzyme. Reactions were brought to 20 µl with water and incubated at 37 °C for at least 4 h after which enzymes were inactivated by 3 µl of stop solution then 10 µl of the product was electrophoresed onto ethidium bromide-containing agarose gel (3%) and the restriction pattern was observed and photographed under UV. Restriction analysis of the wild type sequence of exons was performed using Restriction-Mapper, version 3 (http://restrictionmapper.org).

2.4. Data analysis

Prostate specific antigen (PSA) data were expressed as mean (±SEM). Statistical analysis was performed using GraphPad-Instat software (Graphpad, San Diego, CA, USA). The normal distribution of PSA data was tested by Kolmogorov–Smirnov (KS) normality test. Accordingly, the comparison between means was analyzed by the ANOVA test. *P* value less than 0.05 was considered significant.

3. Results

The study included 2 groups comprising BPH and PCa patients. Their diagnosis was confirmed by the histological analysis (Fig. 1). The PSA data, obtained from patient's medical reports, indicated an increased PSA serum level in BPH patients, where it showed 3–5 fold increase compared to the normal PSA level. PCa patients, however, recorded normal to low PSA levels due to the radical prostatectomy they underwent (Table 1). To investigate the integrity of the restriction pattern of LBD and its possible association with the progression from benign to invasive prostate cancer, we investigated the frequency of LBD polymorphisms, using *SmlI*, *HphI* and *Tsp45I* restriction enzymes in both premalignant and malignant tissues compared to the wild type restriction pattern identified by the Restriction-Mapper.

The LBD consists of 894 pb, extends between nucleotides 2401 and 3294 and has 5 exons (4, 5, 6, 7 and 8) (Fig. 2). Exon 4/5 fragment (424 bp) did not show any *SmlI*, *Tsp45I* or *HphI* restriction abnormalities in both BPH and PCa groups (Fig. 3A). The wild type sequence of exon 6 consists of 134 bp (nt: 2842–2974). This fragment contains a single recognition site for 17 restriction enzymes (including *SmlI* and *Hph1*), whereas *Tsp45I* has no recognition site along the exon sequence. Similar to the wild type, exon 6, in both groups, showed normal *SmlI*, *Hph1* and *Tsp45I* restriction patterns (Fig. 3B). Exon 7, in contrast, demonstrated the constitutional patterns of *SmlI*, *Hph1* and *Tsp45I* only in BPH patients. However, *Hph1* and *Tsp45I* revealed an abnormal pattern in 6 (40%) and 4 (26.7%) of the PCa patients, respectively (Fig. 3, panels CII and CIII). A similar changing pattern was observed in exon 8. This fragment represents the C-terminal domain of the LBD. Its wild type sequence harbors a single recognition site for 17 enzymes and 2 sites for another 5 restriction enzymes. Other known restriction enzymes, however, are non-cutters (do not cleave this fragment). *SmlI*, *Hph1* and *Tsp45I* restriction patterns were conserved in BPH patients (Fig. 3, panel D). However, both *Hph1 and Tsp45I* in PCa patients (group II) showed abnormal restriction patterns in 3 (20%) and 2 (13.3%) in PCa patients, respectively. These patients have a single fragment of 288 bp, due to the elimination of the PhpI restriction site (Fig. 3, panel DII). Also, they have 2 different abnormal banding patterns due to the elimination (or changing) of the *Tsp45I* restriction site (Fig. 3, panel DIII).

4. Discussion

In this work the restriction analysis of *SmlI*, *Tsp45I*, and *HphI* in the LBD of human androgen receptor gene was used to investigate the association between the integrity of this domain and the progression of BPH into PCa. Such approach was previously used to investigate the relation between LBD *StuI* site polymorphism and baldness, where it was found that LBD *StuI* restriction site was detected in 98.1% of young bald men, in 92.3% of older bald men and in 76.6% of non-bald men [13]. Herein, we analyzed the restriction pattern of the LBD in both BPH and (PCa) patients. Many reasons stand behind the choice for the LBD including: (i) This domain is

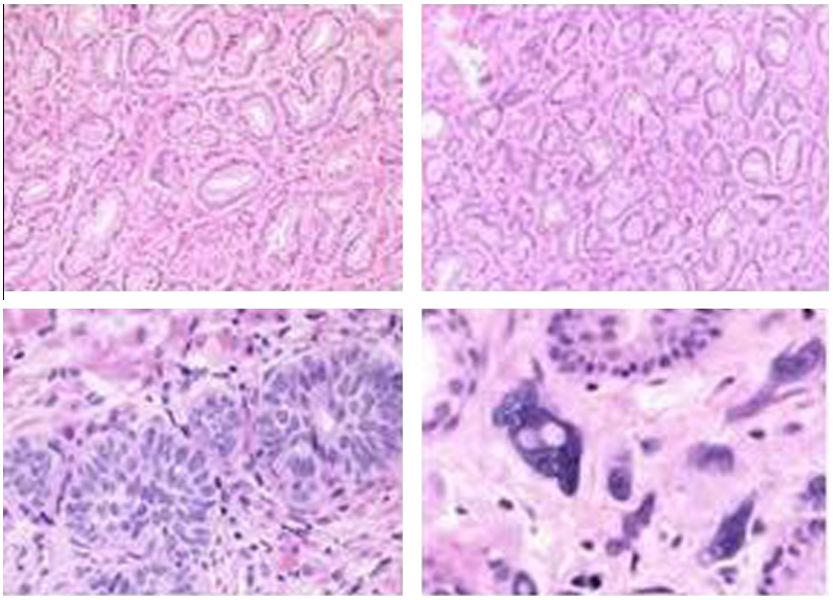

Figure 1 Histological pattern in prostate cancer (top) and benign prostatic hyperplasia patients (bottom).

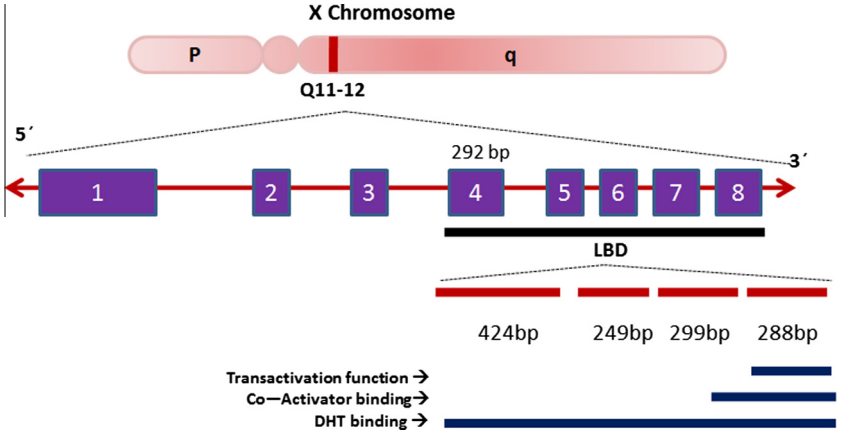

Figure 2 Human androgen receptor gene, located on the X chromosome, at Xq11-12, is encoded by 8 exons (1–8). The figure shows the exons that constitute the receptor ligand binding domain (LBD) and the corresponding amplified fragments used to investigate the restriction polymorphism.

poorly conserved and has hydrophobic nature similar to testosterone or DHT. (ii) Testosterone or DHT binding site, located in this domain, dictates hormone binding specificity [14]. (iii) LBD represents the pocket into which the ligand fits. Consequently, mutations in this domain may affect the conformation of the hormone binding site [15]. (iv) Helix 12 of the LBD plays an important role in the interaction between the androgen receptor and many coactivators, such as Steroid Receptor Coactivator-1 (SRC-1) and antiestrogens, such as tamoxifen [16]. (v) LBD contains the AF-2 of the androgen receptor, which is considered one of the most important functions of the receptor. (vi) LBD contains some domains that represent the binding site for heat shock protein-90 (HSP-90), which maintains the receptor in the nonliganded form in the cytoplasm in absence of the ligand [17].

The approach used is based upon the restriction analysis of 4 fragments representing the entire AR-LBD. The investigated exons (4–8) range in size from 131 bp (exon 6) to 158 bp (exon 7). Exon 4/5 analysis did not show any *SmlI*, *HphI* and *Tsp45I* restriction polymorphism in both benign BPH and PCa patients. Similarly, the 3 sites were conserved in exon 6 derived from both BPH and PCa patients. The normal restriction patterns obtained in both exons 4/5 and 6 do not exclude the existence of point mutations away from the recognition sites of the 3 enzymes used. Previously point mutation (T to G, led to M807R substitution) was reported in exon 6. This mutation has induced complete androgen insensitivity in a patient with 46, XY karyotype and affected the AR transactivation function (Tables 3 and 4) [18].

Exon 7 has kept its restriction integrity in BPH patients. However, *HphI and Tsp45I* site polymorphism was observed in some PCa patients. There genetic abnormalities are supported with the high number of mutations previously reported in exon 7 [19].

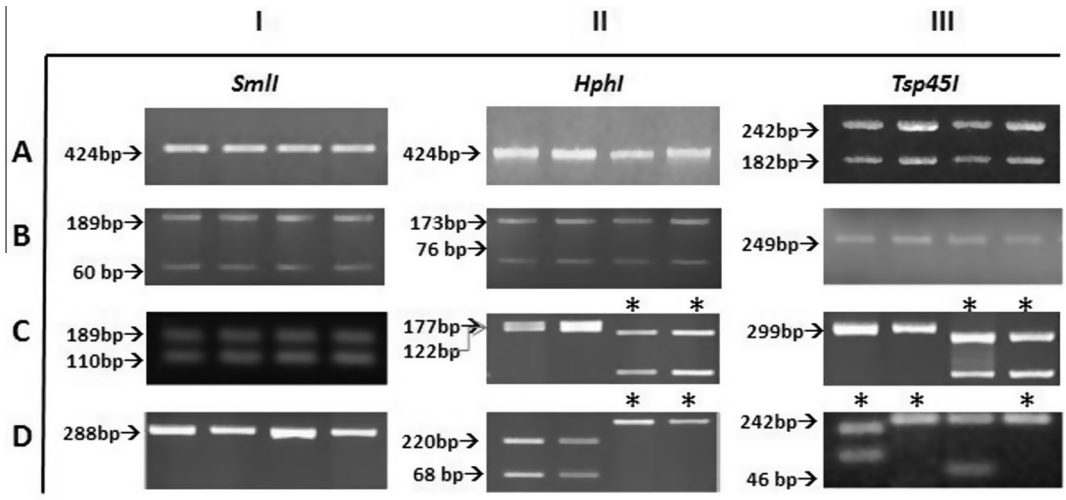

Figure 3 Analysis of human androgen receptor ligand binding domain (AR-LBD) polymorphism by polymerase chain reaction–restriction fragment length polymorphism. DNA samples, derived from benign prostatic hyperplasia and prostate cancer patients, were amplified using exons specific primers. PCR products were digested with the restriction endonucleases *SmlI*, *HphI and Tsp45I* and the digestion products were resolved onto 3% agarose gel with ethidium bromide staining and visualized under UV transilluminator. Panels A–D show the obtained restriction patterns *SmlI*, *Tsp45I*, or *HphI* of exons 4/5, 6, 7 and 8, respectively. Lanes showing an abnormal restriction patterns are marked with *.

Table 3 Recognition sites of *SmlI*, *HphI* and *Tsp45I*, along the wild type sequence of exons 4–8 of AR-LBD.

Enzyme	Recognition sequence	Overhang	Frequency/cut position			
			Exon 4/5	Exon 6	Exon 7	Exon 8
SmlI	CTYRAG	5′	0	1/60	1/110	0
HphI	GGTGA	3′	0	1/76	1/122	1/68
Tsp45I	GTSAC	5′	1/182	0	0	1/46

Y = C or T and R = A or G.

Table 4 Restriction patterns of *SmlI*, *HphI* and *Tsp45I* in exons 4–8 of AR-LBD of benign prostatic hyperplasia and prostate cancer patients.

Exon	Enzyme	Frequency in the wild type sequence	Restriction pattern			
			BPH (*n* = *15*)		PCa (*n* = *15*)	
			Normal	Abnormal	Normal	Abnormal
4/5	*SmlI*	1	15 (100%)	0.0 (0.0%)	15 (100%)	0.0 (0.0%)
	HphI	0	15 (100%)	0.0 (0.0%)	15 (100%)	0.0 (0.0%)
	Tsp45I	0	15 (100%)	0.0 (0.0%)	15 (100%)	0.0 (0.0%)
6	*SmlI*	1	15 (100%)	0.0 (0.0%)	15 (100%)	0.0 (0.0%)
	HphI	1	15 (100%)	0.0 (0.0%)	15 (100%)	0.0 (0.0%)
	Tsp45I	0	15 (100%)	0.0 (0.0%)	15 (100%)	0.0 (0.0%)
7	*SmlI*	1	15 (100%)	0.0 (0.0%)	15 (100.0%)	0 (0.0%)
	HphI	1	15 (100%)	0.0 (0.0%)	6 (40%)	9 (60.0%)
	Tsp 45I	0	15 (100%)	0.0 (0.0%)	3 (20%)	12 (80.0%)
8	*SmlI*	0	15 (100%)	0.0 (0.0%)	15 (100%)	0 (0.0%)
	HphI	1	15 (100%)	0.0 (0.0%)	4 (26.7%)	11 (73.3%)
	Tsp45I	1	15 (100%)	0.0 (0.0%)	2 (13.3%)	13 (86.7%)

Similarly, BPH patients did not show *Tsp45I* resection polymorphism in exon 8, where *Tsp45I* did not cleave the amplified fragment (288 bp). The androgen receptor gene mutations database [20], has reported tens of mutations along exon 8. Also, some reports identified Q919R mutation in a prostate cancer patient. This may explain the development of *Tsp45I* polymorphism in 13% of PCa patients. The observed fragment (~150 bp) may indicate a newly developed restriction site due to point mutation(s). Moreover the wobbling, coding of an amino acid with more than one codon, *Tsp45I* site (GTSAC) may enhance the chance of the development of a new cleavage site. Clustering of these genetic alterations in the last C-terminal part of the receptor (exons 7 and 8) is translated as defects in testosterone binding to the receptor, co-activator interaction and transactivation function due to the localization of these functions along or in the C-terminal part of LBD (Fig. 2). This may explain how prostate cancer is transformed and progresses from an androgen-dependent state to an androgen-independent state [21]. Although androgen deprivation therapy suppresses the AR activity and inhibits the growth of prostate cancers, such genetic alterations could be the reason of recurrence and the development of androgen independent progression to prostate cancer [22].

5. Conclusion

The data obtained may present an example for the parallel association between the existence of restriction polymorphism and the progression of benign prostatic hyperplasia to prostate cancer. Although normal restriction analysis does not eliminate the possibility of mutations, this approach presents a simple and fast genetic based discrimination between benign hyperplasia and invasive prostate cancer.

Conflict of interests

The authors report no conflicts of interest.

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References

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008, cancer incidence and mortality worldwide. Int Agency Res Cancer 2010.
[2] Jang SJ, Mao L. Methylation patterns in human androgen receptor gene and clonality analysis. Cancer Res 2000;60(4):864–6.
[3] Shand RL, Gelmann EP. Molecular biology of prostate-cancer pathogenesis. Curr Opin Urol 2006;16:123–31.
[4] Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes Dev 2000;14(19):2410–34.
[5] Epstein JI. Precursor lesions to prostatic adenocarcinoma. Virchows Arch 2009;454(1):1–16.
[6] Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin 1972;22:232–40.
[7] Lim Ji Hyae, Kim ShinYoung, Lee Si Won, Park So Yeon, Chung Jung Yeol, Kim Moon Young, et al. Association between genetic polymorphisms in androgen receptor gene and the risk of preeclampsia in Korean women. J Assist Reprod Genet 2011;28 (1):85–90.
[8] Ma JG, Li WP, Jiang YQ. Androgen receptor mutation and progression of prostate cancer. Zhonghua Nan Ke Xue 2011;17 (7):649–54.
[9] Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 1988;240(4850):324–6.
[10] Brinkmann AO, Klaasen P, Kuiper GG, van der Korput JA, Bolt J, de Boer W, et al. Structure and function of the androgen receptor. Urol Res 1989;17(2):87–93.
[11] Saporita AJ, Zhang Q, Navai N, Dincer Z, Hahn J, Cai X, et al. Identification and characterization of a ligand-regulated nuclear export signal in androgen receptor. J Biol Chem 2003;278 (43):41998–4205.
[12] Wurtz JM, Bourguet W, Renaud JP, Vivat V, Chambon P, Moras D, et al. Canonical structure for the ligand binding domain of nuclear receptors. Nat Struct Biol. 1996;3:206.
[13] Ellis JA, Stebbing M, Harrap SB. Polymorphism of the androgen receptor gene is associated with male pattern baldness. J Invest Dermatol 2001;116(3):452–5.
[14] Mangelsdorf DJ, Thummel C, Beato M. The nuclear receptor superfamily: the second decade. Cell 1995;83:835–9.
[15] Bourguet W, Ruff M, Chambon P. Crystal structure of the ligand-binding domain of the human nuclear receptor RXRα. Nature 1995;375:377–82.
[16] Brzozowski AM, Pike AC, Dauter Z. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389: 753–8.
[17] Denis M, Gustafsson JA, Wikström AC. Interaction of the Mr = 90,000 heat shock protein with the steroid-binding domain of the glucocorticoid receptor. J Biol Chem 1988;263(34):18520–3.
[18] Adeyemo Oyewole, Kallio Pekka J, Palvimo Jorma J, Kontula Kimmo, Jänne Oill A. A single-base substitution in exon 6 of the androgen receptor gene causing complete androgen insensitivity: the mutated receptor fails to transactivate but binds to DNA in vitro. Hum Mol Genet 1993;2(11):1809–12.
[19] Brinkmann AO, Jenster G, Ris-Stalpers C, van der Korput JA, Brüggenwirth HT, Boehmer AL, et al. Androgen receptor mutations. J Steroid Biochem Mol Biol 1995;53(1–6):443–8.
[20] Gottlieb Bruce, Lehvaslaiho Heikki, Beitel Lenore K, Lumbroso Rose, Pinsky Leonard, Trifiro Mark. The androgen receptor gene mutations database. Nucleic Acid Res 1998;26(1):234–8.
[21] Cheng Y, Yu Pan, Duan Xiuzhi, Liu Chunhua, Xu Siqi, Chen Yuhua, et al. Genome-wide analysis of androgen receptor binding sites in prostate cancer cells. Exp Ther Med 2015;9(6):2319–24.
[22] Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. N Engl J Med 2004;351:1488–90.