

Androgen Receptor as a Potential Sign of Prostate Cancer Metastasis

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BACKGROUND. Androgen receptor (AR) expression and its modulation through the carcinogenesis process have been investigated in several studies with conflicting results.

MATERIALS AND METHODS. In situ hybridization and immunocytochemistry were used to examine AR expression in prostatic needle core biopsies of benign, high grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma.

RESULTS. A significant increase in AR mRNA levels was found in the cancerous prostatic cells when compared with the benign tissue biopsies. AR abundance in HGPIN was found to be almost half-way between that observed in benign and in cancerous tissue. In the benign prostatic epithelium, the immunocytochemistry data show that AR is exclusively expressed in the nuclei of epithelial cells. However, in 72% of examined cancer biopsies, AR was expressed in both the cytoplasm and nuclei. After examination of medical records of 100 patients diagnosed with prostate cancer, it was found that the AR was expressed in both cellular compartments of cancer cells in 81% of cases when cancer was found to have metastasized outside the prostate. In contrast, when the cancer was organ-confined, AR was localized in both the nuclei and cytoplasm in only 66% of cases. Moreover, when the AR was expressed in the cytoplasm of cancerous cells, consecutive serial sections immunostained with the mitochondrial marker suggest that AR is localized in the mitochondria.

CONCLUSIONS. AR mRNA expression is significantly higher in prostate cancer when compared to benign prostatic tissue. *Prostate* 69: 1704–1711, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: androgens; prostatic intraepithelial neoplasia (PIN); immunohistochemistry; in situ hybridization; mitochondrial marker

INTRODUCTION

Androgens (testosterone and dihydrotestosterone (DHT)) are known to play a central role in the growth and proliferation of both the normal and pathological prostate [1]. It is now well-recognized that 50–75% of intraprostatic androgens originate from the testis. The remaining 25–50% of androgens are produced locally in the prostate tissue by the intracrine transformation of the adrenal precursors dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) [2–4]. Both the testicular and adrenal sources of androgens produce DHT in the prostate, a steroid that acts through a unique androgen receptor (AR).

AR is a transcription factor that belongs to the steroid hormone receptor superfamily. The activated ligand-bound AR translocates to the nucleus to activate or

suppress androgen-sensitive genes. In some studies, in the absence of ligand, AR remains in the cytoplasm [5]. The localization of AR in the prostate as well as its modulation through the carcinogenesis process have been investigated in several studies. Conflicting results, however, have been obtained [6–13]. For example, Sweat et al. [10] found that AR was expressed at a lower level in prostate cancer when compared with benign tissue while Cardillo et al. [11] have observed a higher

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level of AR in prostate cancer. In our laboratory, when immunohistochemistry was used to examine biopsies of benign, high-grade PIN and prostatic adenocarcinoma, no significant difference was found in the number of labeled nuclei nor in their staining intensity [14].

Previously, when immunostaining was used to study AR in any tissue, cytoplasmic localization was exclusively considered as a background. However, in some recent studies, cytoplasmic labeling has been demonstrated to be specific. In our laboratory, examination of mouse skin sebaceous glands revealed AR cytoplasmic localization in gonadectomized animals. However, when these animals received DHT for 3 weeks, AR localization was completely shifted to the nuclei [5]. Moreover, AR was found to be localized in the cytoplasm of cultured prostate cancer cells [15] and a splicing variant of AR was detected in the cytoplasm of metastatic prostate cancer cells [16]. As indicated by Diallo et al. [17], cytoplasmic AR could potentially be used as an independent predictor of biochemical recurrence.

In different cell types, the localization of various steroid receptors in mitochondria has been demonstrated [18] and AR was detected in the mitochondria of human sperm and LNCaP cells [15]. An electron microscope study demonstrated an increase of mitochondrial number and size in prostate cancer compared to BPH cells [19].

In an attempt to find out if there is a different expression level of AR mRNA between normal and diseased prostatic cells, we have used *in situ* hybridization to quantify the mRNA level in benign prostate, high-grade PIN and prostate cancer. We have also used immunocytochemistry to examine the intracellular localization of AR in normal and diseased human prostate. Moreover, in consecutive serial sections, we

have used an antibody raised against a 60 kDa non-glycosylated protein component of mitochondria as a mitochondrial marker.

MATERIALS AND METHODS

Tissue Preparation

All prostate specimens used in this study were kindly provided by Dr. Martin Lemay and Dr. Michel Tremblay (Centre Hospitalier de l'Université Laval, CHUL, Quebec City, Quebec, Canada). Biopsies were collected between the years 1991 and 1998 from patients without hormone therapy aged between 50 and 79 years. A total of 150 different paraffin-embedded benign and cancerous archival human prostate needle core biopsies were used for histological sections. Prostate cancer patients were free of any hormonal treatment and their biopsies were used to establish prostate cancer diagnosis. In sections where high-grade PIN acini identification was uncertain, in consecutive serial sections, we have used high molecular weight cytokeratin (HMWCK) antibody as a specific marker of basal cells to ascertain the absence or the disrupted basal cell layer. AR has been studied by *in situ* hybridization in 29 biopsies and by immunocytochemistry in 150 biopsies. The mitochondrial marker was examined in 26 biopsies. To find out if the expression of AR in the cytoplasm has any prognostic value, the medical reports and the patient-following up data of all patients diagnosed with prostate cancer were re-examined by the specialist (Dr. Leonello Cusan). For clinical staging, adenocarcinomas were classified according to the Jewett-Whitmore staging system (Table I) [20]. The highest stage reached after prostate cancer diagnosis was used to establish the prognostic value.

TABLE I. Distribution of the Number and Percentage of Prostate Cancer Patients According to the Jewett-Whitmore Clinical Staging System

	Stage	AR in the nuclei	AR in both the nuclei and cytoplasm	
		Number of patients	Number of patients	%
Adenocarcinoma organ-confined (59)	A	1	0	
	B0	1	2	
	B1	10	22	
	B2	8	15	
	Sub total	20	39	66
Adenocarcinoma spread outside the prostate (41)	C1	5	11	
	C2	2	10	
	D1	0	3	
	D2	1	9	
	Sub total	8	33	81

Immunocytochemistry

Immunostaining was performed using Zymed SP kits (San Francisco, CA). To confirm high-grade PIN identification, consecutive serial sections of 3 μm were cut for each specimen and immunostained with anti-HMWCK. Microwave antigen retrieval technique using citrate buffer was performed for all antibodies [21]. Sections were incubated at room temperature with one of the following antibodies: rabbit anti-AR (1:500) (N-20, Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 hr, mouse anti-HMWCK (1:50) (Dako Canada, Inc., Mississauga, Ontario) for 30 min, or mouse anti-mitochondria (prediluted) (Abcam, Inc., Cambridge, MA) for 50 min. Slides were then washed in PBS buffer and incubated with a biotinylated secondary antibody for 10 min and then with streptavidin–peroxidase for another 10 min. Diaminobenzidine was used as the chromogen to visualize the biotin/streptavidin–peroxidase complex, under microscope monitoring. For HMWCK and the mitochondrial marker negative controls, mouse IgG's (Vector Laboratories, Burlingame, CA) were used at the same dilution as that of the antibody. Moreover, as a control for AR, the antibody was preincubated with an excess of its immunizing peptide before its application on the sections.

Evaluation of AR Labeling Localization

To determine the precise localization of AR in benign and cancerous prostatic cells, the whole biopsy was examined under an optical microscope at 400 \times . Two experienced investigators, who had no knowledge of the clinical data, evaluated the slides of all biopsies. All the tissue samples were examined. The biopsies were classified whether or not a nuclear reaction or a nuclear and a cytoplasmic reaction was present. All cytoplasmic immunoreactions were considered by the observers, including light labeling intensity.

In Situ Hybridization

The vector used for production of the cRNA probe was constructed by insertion into a pCR-Blunt II-TOPO (Invitrogen, Ontario, Canada) of a 703 bp human AR (Genebank No. NM_000044) cDNA fragment. Labeled sense and antisense cRNA probes were synthesized by incubation of linearised template (250 μg) with 100 μCi [^{35}S]UTP (1,000 Ci/mmol; GE Healthcare, UK) in the presence of T7 or Sp6 RNA polymerase for 60 min at 37°C, according to the manufacturer's recommendations (Riboprobe Combination System; Promega, WI). Consecutive serial sections of 4 μm were cut for each specimen. Slides were deparaffinized in toluene, rehydrated through graded alcohols, treated with proteinase K (1 $\mu\text{g}/\text{ml}$ in 100 mM Tris–HCl, 50 mM

EDTA, pH 8.0) for 30 min at 37°C and with acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0) for 10 min, then dehydrated through graded alcohols. After drying the sections, a prehybridization buffer was applied (50% formamide, 300 mM NaCl, 10 mM Tris–HCl at pH 8.0, 1 mM EDTA, 1 \times Denhardt's solution, 200 $\mu\text{g}/\text{ml}$ tRNA and 8% dextran sulfate) for 2 hr at room temperature. Hybridization was then performed overnight using hybridization buffer (prehybridization buffer plus 50 mM DTT and 2 \times 10⁶ cpm of ^{35}S -labeled RNA/slide) at 53°C. Excess probes were removed by washing in 50% formamide, 2 \times SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) at 58°C for 90 min, followed by 20 $\mu\text{g}/\text{ml}$ Rnase A in NTE buffer (500 mM NaCl, 10 mM Tris–HCl and 5 mM EDTA, pH 8.0) for 30 min at 37°C and then another 50% formamide, 2 \times SSC at 58°C for 90 min. After dehydration in graded alcohols and air drying, the slides were dipped in Hypercoat LM-1 emulsion (GE Healthcare) and exposed for 21 days in the dark at 4°C. The slides were then developed at 20°C in Kodak D-19 developer for 7 min, fixed for 4 min in Kodak Rapid Fixer and counterstained with hematoxylin and eosin. Hybridization with a sense probe was used as the negative control and only background signal was detected in all the slides.

Image Analysis

For the measurements of silver grain density after the in situ hybridization, a 10 \times objective was used to capture 1–5 images from each of the used prostate biopsies. Images were captured with a DC-330 3CCD color camera (Dage-MTI, USA) and quantified using Image-Pro Plus 3.0 software (Media Cybernetics, USA). To prevent measuring the background silver grains of in situ hybridization labeling, the mean value of silver grain density was measured in a similar area of the sense control consecutive section and subtracted from the mean value of silver grain density calculated from the anti-sense slides. The mean value of silver grain density is the integrated optical density per area (OD).

Statistical Analysis

Data are expressed as the means \pm SEM. Statistical significance was determined according to the multiple-range test of Kramer [22].

RESULTS

Androgen Receptor mRNA

Using a ^{35}S -labeled probe, AR mRNA expression was investigated in benign prostate, high-grade PIN as well as in prostatic adenocarcinoma. In benign prostatic epithelium, a weak autoradiographic signal was

detected (Fig. 1A,d) while the signal was found to be at a higher level in high-grade PIN (Fig. 1A,e) and much higher in prostatic adenocarcinoma biopsies (Fig. 1A,f). When the hybridization was performed with the sense probe as a control, only scattered silver grains were detected over the epithelium (Fig. 1A,g,h,i). Image analysis confirmed the visual examination of the silver grains by showing that AR mRNA levels are significantly higher in prostate cancer when compared to the benign prostate ($P < 0.01$). The difference observed

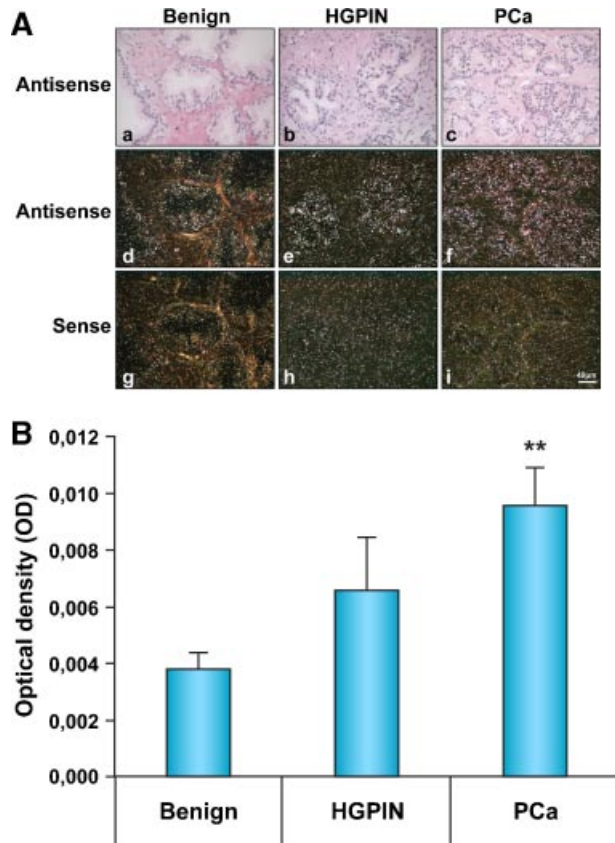


Fig. 1. Expression of the AR mRNA by in situ hybridization in paraffin sections of human prostate. **A:** In the upper row, **a**, **b**, and **c** are bright field images re-captured using the dark field to show the silver grains as seen in **d**, **e**, and **f**, respectively. Consecutive serial sections of benign human prostate (**d** and **g**), high-grade PIN (HGPN) (**e** and **h**) and prostate cancer (PCa) (**f** and **i**) hybridized in situ with ^{35}S -labeled AR antisense (**d**, **e**, and **f**) and sense (**g**, **h**, and **i**) riboprobes. A weak autoradiographic signal is detected in benign prostatic epithelium (**d**), while AR expression increases in HGPN (**e**) and in PCa biopsy (**f**). Only scattered silver grains can be detected as a background when the sense probe was used as a control (**g**, **h**, and **i**). **B:** Comparison between the optical density of silver grains which represent AR expression in benign prostatic epithelium ($n = 10$), HGPN ($n = 5$) and PCa ($n = 14$). A gradual increase is observed in HGPN and prostate cancer and the OD of prostate cancer is significantly higher than that of benign prostate. Data are presented as means \pm SEM. $**P < 0.01$.

between benign and high-grade PIN or between prostate cancer and high-grade PIN was not statistically significant (Fig. 1B).

Androgen Receptor Localization in the Cytoplasm

The intracellular localization of the AR protein was determined by immunocytochemistry in a total of 150 biopsies. Out of 100 examined prostate cancer biopsies, AR was expressed in the nuclei as well as in the cytoplasm in 72 of them (Fig. 2, a as example), while in adjacent noncancerous acini of the same needle core biopsy sections (Fig. 2b as example), AR labeling was

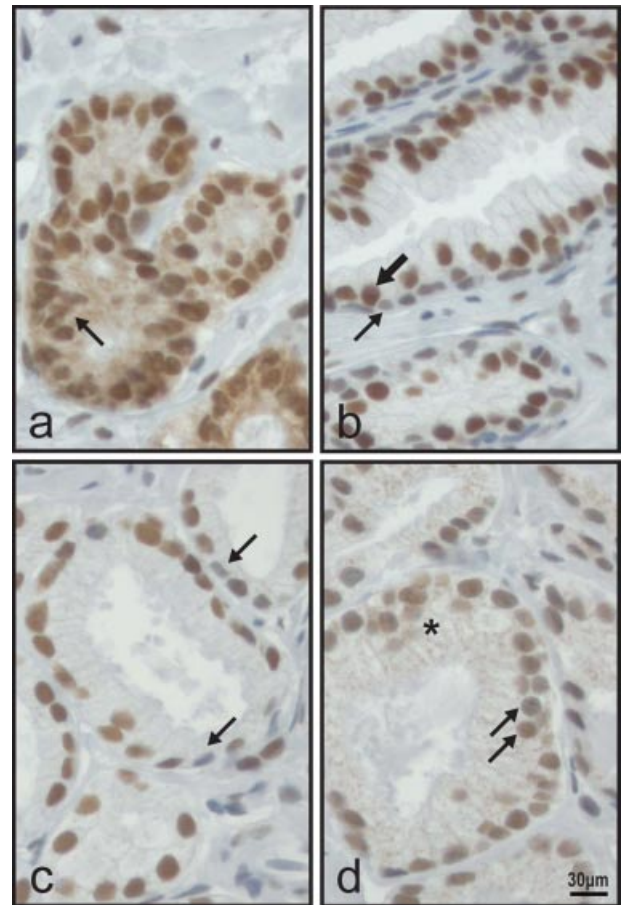


Fig. 2. Expression of the AR protein by immunocytochemistry. In cancerous acini, AR immunolabeling was detected in the nuclei as well as in the cytoplasm (arrow in **a**), while in adjacent noncancerous acini of the same needle core biopsy section (**b**), AR labeling was exclusively detected in the nuclei and the staining intensity was higher in luminal cells (thick arrow) when compared to basal cells (thin arrow). In cancer cells, the labeling intensity of AR in the nuclei and in the cytoplasm varied in the same biopsy section from unlabeled nuclei and cytoplasm (arrows in **c**) to detectable labeling in both compartments (arrows in **d**). Granular labeling, could be observed after careful examination of the AR immunoreaction in the cytoplasm of cancer cells (asterisk in **d**).

exclusively detected in the nuclei. In fact, no AR cytoplasmic reaction was detected in noncancerous acini or in any of the benign biopsies. A cytoplasmic reaction was detected, however, in high-grade PIN acini. The labeling intensity of AR in the nuclei and in the cytoplasm of cancer cells varied within the same biopsy section and also between biopsies of different patients from unlabeled nuclei and cytoplasm (Fig. 2c) to detectable labeling in both compartments (Fig. 2d).

After examination of medical reports of all 100 prostate cancer patients, it was found that the AR is expressed in both the cytoplasm and nuclei of cancer cells in 81% of the cases when prostate cancer cells were found to be spread outside the prostate (Table I). When the cancer was organ-confined, AR was localized in both the nuclei and cytoplasm in only 66% of the cases. When we examined the percentage of patients at the advanced stage D of metastasis amongst all prostate cancer patients, only one out of thirteen had AR expression limited to the nuclei (Table I and Fig. 3). No correlation was found with the Gleason score or the PSA level.

Androgen Receptor and Mitochondria

The cytoplasmic localization of AR was determined with the aid of a mitochondrial marker in consecutive serial sections. The mitochondrial marker immunoreaction was observed as a clear granular labeling in all prostate cells. These granules, however, were small in noncancerous acini while being larger and more numerous in high-grade PIN and in prostate cancer acini. Examination of consecutive serial sections

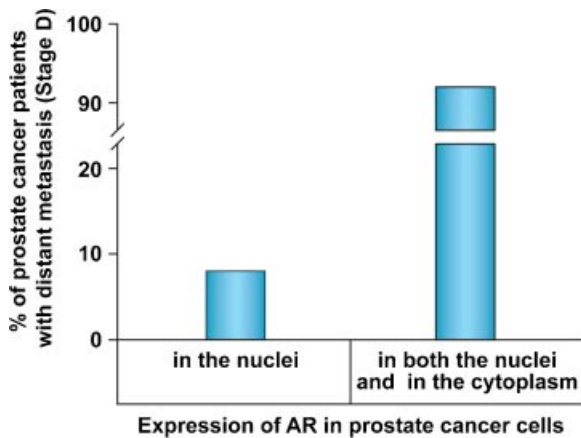


Fig. 3. Comparison of the percentage of patients with distant metastasis to all stage D prostate cancer patients in relation to the intracellular localization of AR. In patients where AR is expressed exclusively in the nuclei, the percentage of patients with metastasis was found to be 8% (1 out of 13). Meanwhile, this percentage reached 92% when AR was detected in both the cytoplasm and nuclei of cancer cells (12 out of 13).

immunostained for the mitochondrial marker and AR revealed a similar staining pattern of both markers in the cytoplasm and indicated that AR is localized in both the nuclei and mitochondria (Fig. 4, inset in a and b). In prostate cancer biopsies, various staining intensities

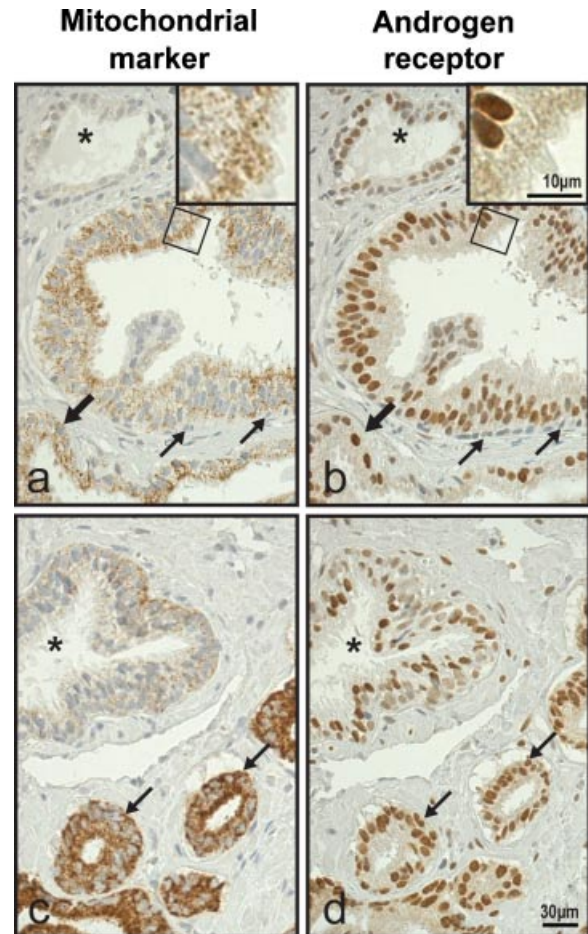


Fig. 4. Immunostained sections with antibodies against a mitochondrial marker (a and c) while their consecutive serial sections are immunostained against AR (b and d). A large acinus of HGPIN with some remaining basal cells could be seen underlying the stratified columnar cells (small arrows in a and b). Noncancerous acini (asterisks in a–d) with continuous layers of basal cells underlying the luminal cells could be seen in the vicinity of cancerous acini where basal cells are absent (large arrow in a and b and small arrows in c and d). The mitochondrial marker showed variable immunostaining intensity reaction between noncancerous acini (asterisks in a and c) as well as between cancerous acini of different biopsies (thick arrow in a and arrows in c). However, the labeling intensity of noncancerous acini was always much less when compared to HGPIN and cancerous acini (a and c). In the consecutive serial sections immunostained against AR, careful examination of the cytoplasm of HGPIN and cancerous cells (b and d) reveals fine but weak stained granules similar to the mitochondrial marker immunostaining. However, when examined at higher magnification (inset in a and b), similar labeled mitochondria are clearly detected in the cytoplasm of the two consecutive serial sections.

of the mitochondrial marker labeling were observed, from a moderate (Fig. 4a) to a strong (Fig. 4c) labeling. Moreover, the labeling detected in noncancerous acini was variable as seen in Figure 4 (asterisks in a and c), but it was always lower in its intensity when compared to malignant acini. A strong expression, however, of the mitochondrial protein in prostate cancer acini was not always accompanied by a strong presence of AR in the mitochondria (Fig. 4).

DISCUSSION

The present study shows that while immunostaining failed to show differences of AR protein expression, *in situ* hybridization revealed a gradual increase in AR mRNA level from benign prostates to HGPIN and then to prostate cancer, with a level of expression significantly higher in prostate cancer compared to benign tissue. The conclusions of previous studies regarding the modulation of AR expression with cancer progression are contradictory. Thus, in a previous study, using immunocytochemistry, we did not observe a significant difference in the number of AR-labeled nuclei or in their staining intensity among all the examined biopsies of benign and malignant prostates [14]. However, as mentioned above, other studies have suggested that AR is at a higher expression level in benign prostatic epithelium than in PIN and cancer [10] while others concluded that AR immunoreactivity significantly increased from BPH to PIN to prostatic carcinoma [11].

This discrepancy could be explained by the quality of the biopsies used in relation with the time elapsed between their collection and fixation, to the variable immunostaining characteristics of the Santa Cruz polyclonal anti-AR antibody used and/or to the known limitations of the immunostaining technique. Another cause of discrepancy could be that, in some studies, comparison of AR expression was analyzed between noncancerous and cancerous tissues of the same patient; noncancerous acini of a diseased prostate might be different if compared with the acini of a healthy prostate. Recently, Fujimoto et al. [23] have suggested that prostate cancer cells change androgen-sensitivity through overexpression of AR which could result in transition from androgen-dependent to apparently androgen-independent prostate cancer cells.

Recent studies in other cell types have shown, in the absence of ligand, that AR is mostly localized in the cytoplasm and moves to the nuclear compartment after androgen treatment [5,24]. Moreover, cytoplasmic staining was observed in prostatic carcinoma metastatic cells only in patients who had received anti-androgenic therapy [7] while in other studies, AR

expression was restricted to the cytoplasm of HGPIN but not in benign epithelium or cancer [9,10]. It has also been suggested that AR has a tendency to aggregate in the cytoplasm when overexpressed and, to an even greater extent, as demonstrated by a polyglutamine-expanded AR, with a mutant AR containing 48 glutamines [25]. These previous observations could be in agreement with our results in the sense that the overexpressed AR in prostate cancer cells might lead to its cytoplasmic accumulation. The precise role of AR cytoplasmic accumulation in the prostate cancer cells remains unclear.

Although the expression of AR and its involvement in prostate cancer has been studied for several years, its possible prognostic significance in prostate cancer was only recently investigated [17,26–28]. Several studies have demonstrated that AR could have a prognostic value for patients at high risk of disease relapse after radical prostatectomy. Some of these studies suggest that cytoplasmic AR could contribute to androgen-independent prostate cancer [17,26,28]. Moreover, Morgan et al. [29] have shown that the cutaneous fatty acid-binding protein (C-FABP) could be used as a potential prognostic marker to predict patient outcome and survival time. Meanwhile, it has been demonstrated that this above-mentioned protein could induce metastasis through the up-regulation of other oncogenes such as vascular endothelial growth factor [30] and through its ability to bind and transport fatty acids [31]. In the present study, in order to study the clinical relevance of AR in the cytoplasm of prostate cancer cells, we have examined the medical reports and follow-up data of all patients. Interestingly, when the AR was expressed in the cytoplasm and nuclei of localized prostate cancer, the possibility of cancer metastasis increased compared to patients having AR only expressed in the nuclei. In a preliminary study, we examined about half the number of these patients. Later on, we increased the number and the results were very similar. More research is required to understand the relationship between cytoplasmic AR and prostate cancer metastasis.

Using immunostaining, cytoplasmic AR appears as fine granular staining, suggesting that AR could be localized in the mitochondria. Using consecutive serial sections and a specific mitochondrial marker, we have observed that the AR immunolabeling reaction co-localized with the mitochondrial marker. These results are consistent with another study where AR was found in the mitochondria of LNCaP human prostate cancer cells and human sperm [15]. After careful examination of the mitochondrial marker in all our biopsies, it is interesting to observe that the labeling in general was much stronger, the granules larger and longer in the cytoplasm of HGPIN and cancerous acini

compared to the benign acini of the same patient or other healthy patients. These differences in the staining intensity between the cancerous and non-cancerous acini were observed with ease even at a low magnification, thus indicating an increase in mitochondrial size and number in cancer cells and suggesting that this mitochondrial marker could be useful as an additional marker of prostate cancer. In agreement with our findings, in an electron microscopy study, Mao et al. [19] have shown that the number of mitochondria increased in prostate cancer cells and that some cancer cells were packed with enlarged or distended mitochondria when compared with benign tissue [19]. It is known, on the other hand, that during malignant transformation, which usually takes place in the peripheral zone of the prostate, cells switch from energy inefficient benign cells to energy efficient tumor cells [32]. This change is accompanied by a major change in mitochondrial metabolic function and overexpression of some mitochondrial proteins such as α -methyl acyl-CoA racemase (AMACR), mitochondrial intermediate peptidase (MIPEP), serine hydroxymethyltransferase 2 (SHMT2) and cyclooxygenase-2 [33–35]. In the present study, using an indirect method we have observed the overexpression of AR in the mitochondria of cancer cells. Therefore, the morphological alterations in the mitochondria of cancer cells could be explained by these recent findings of changes of the mitochondrial function and its proteins. However, the function of mitochondrial AR remains unclear. Further studies should be performed on mitochondrial proteins to confirm AR localization as well as its mitochondrial function.

CONCLUSION

In conclusion, AR mRNA expression is significantly higher in prostate cancer compared to benign prostatic tissue. When the AR is localized in the mitochondria of prostatic cancer cells, the possibility of prostate cancer metastasis appears to be higher. Thus, AR intracellular localization could potentially be used as a prognostic marker of metastasis while the examined mitochondrial marker could be used as an additional marker of prostate cancer.

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