

Distant Metastases from Prostatic Carcinoma Express Androgen Receptor Protein¹

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ABSTRACT

Nearly all primary prostatic carcinomas have been found to express the androgen receptor (AR) protein, which is the intracellular mediator of androgen action. To gain a better insight into the mechanisms of androgen independence of advanced prostatic carcinoma, it is important to know whether the AR is also present in metastases of androgen-independent tumors. We have assessed the status of the AR and the prostate-specific antigen in 22 metastases of 18 patients with progressive prostate cancer. In 18 cases, the metastases were localized in bone, in 3 cases in the epidural space, and in 1 case in the periosteum. All but one patient had received some kind of endocrine treatment for prostatic carcinoma. Paraffin-embedded tissue sections were stained for the AR following a streptavidin-biotin-peroxidase protocol with the polyclonal antibody PG-21, which is directed against amino acids 1 through 21 of the rat and the human AR. The percentage of AR-positive cells was evaluated on the basis of an arbitrary 4-point scale. All 22 tumor metastases displayed AR positivity. One AR-positive metastatic lesion did not stain for prostate-specific antigen, but in all other metastases, this protein was detected by means of immunohistochemistry. The present study provides evidence that, unlike androgen-independent prostatic carcinoma cell lines, distant prostatic carcinoma metastases do express the AR. These findings indicate that the AR may be involved in the progression of prostate cancer.

INTRODUCTION

At the time of diagnosis, more than 50% of prostatic carcinomas are no longer confined to the organ. In nearly one-half of the cases, distant metastases will involve bone (1). Preferential metastatic spread to bone has been attributed to various factors produced by bone cells (2).

Metastatic carcinoma of the prostate is generally treated with some kind of endocrine therapy, which either lowers the level of circulating androgen (surgical castration or administration of luteinizing hormone-releasing hormone analogues) or inhibits the function of the AR³ (antiandrogens). Frequently, these two therapeutic approaches are combined (3). However, the beneficial effects of this therapy are time limited. On this account, the primary tumors of patients with advanced prostatic carcinomas have been investigated for possible alterations in the transmission of androgen signals that are associated with tumor progression. These studies were focused on the expression and structure of the AR (4-9). The majority of progressive prostatic carcinomas contain cell populations that, on immunohistochemistry, are positive for the AR (4, 5). Several AR mutations in prostatic carcinomas have been detected (6-9). In two studies, AR mutation has been described as a rare event in primary tumors (6, 7). However, a recent study by Gaddipati *et al.* (8) reports the frequent appearance of a point mutation at codon 877 in patients with advanced prostatic carcinomas, and Suzuki *et al.* (9) have detected the same mutation in three tumor metastases obtained from one patient.

To date, AR expression in distant metastases from prostatic carcinoma has not been extensively studied. Some data available on this subject were collected in studies on the two human prostatic

carcinoma cell lines, PC-3 and DU-145. The growth pattern of both cell lines, which were derived from tissues other than nodal metastases (10, 11), is androgen independent. PC-3 cells were derived from a bone metastasis of a prostatic carcinoma patient (10). They were found to be poorly differentiated, to exhibit no androgen-regulated secretion of PSA protein, to contain very low levels of wild-type AR mRNA as assessed by means of the PCR, and to be highly metastatic (10, 12-15). DU-145 cells, which were obtained from a brain metastasis of a prostatic carcinoma patient, were found to be poorly differentiated, devoid of AR, and somewhat less aggressive than PC-3 cells (11, 12, 14, 15). To gain more insight into AR expression in metastatic lesions, we have performed a study on 22 specimens obtained from metastases of prostatic carcinoma patients.

MATERIALS AND METHODS

Tissue Samples. Twenty-two metastases from prostatic carcinoma were obtained from 18 different patients. The metastatic lesions were located in bone (18 cases), in the epidural space (3 cases), and in the periosteum (1 case). The specimens were obtained by surgical intervention performed either at the Department of Neurosurgery or at the Department of Orthopedics of Innsbruck University. Operations were performed primarily to provide some relief in the region affected by the metastatic process. Primary tumor specimens were not available at the time when the metastatic samples were obtained. None of the patients had been subjected to radical prostatectomy. All but one patient had received some kind of endocrine therapy preoperatively. Three patients had received diethylstilbestrol after orchiectomy several years before. This therapeutic regimen, however, is no longer used at our institution. Four patients had to be operated on twice, and metastatic specimens were obtained on both occasions.

Prior to histopathological examination, the bone samples were routinely fixed in 10% formalin, decalcified by 3-chloro-acetic acid, embedded in paraffin, cut at 5- μ m thickness, and stained with hematoxylin and eosin. All metastases were evaluated by the same pathologist (A. Hi.). The tumors were graded according to WHO recommendations (16).

Antibodies. The polyclonal anti-AR antibody PG-21 (Paesel-Lorei, Frankfurt/M, Germany) that is directed against amino acids 1 through 21 in the NH₂-terminal region of the AR was used (17). This antibody does not cross-react with estrogen, progesterone, and glucocorticoid receptors. The secondary antibody applied for AR staining was biotinylated rabbit anti-mouse IgG (Dako). PSA staining was performed with a polyclonal antibody (Dako, Glostrup, Denmark) and a peroxidase-labeled swine anti-rabbit secondary antibody (Dako).

Immunohistochemistry. The tissue sections were deparaffinized in xylene and rehydrated in a series of alcohol solutions. After 10 min of microwave irradiation in citrate buffer, endogenous peroxidase was blocked with sodium azide, glucose, and glucose-oxidase (all reagents were obtained from Sigma, Deisenhofen, Germany).

AR staining was performed according to a streptavidin-biotin-peroxidase protocol. The antibody PG-21 (1:10 dilution) was applied to the tissue sections inside a humidified chamber at room temperature for 60 min. After two washes in PBS, the biotinylated secondary antibody (1:500 dilution) was applied at room temperature for 30 min, followed by peroxidase-labeled streptavidin (1:800 dilution) for 30 min. For PSA staining, no microwave pretreatment was performed. The primary anti-PSA antibody was applied for 60 min at a dilution of 1:800 followed by the above-mentioned peroxidase-labeled swine anti-rabbit antibody. The enzymatic reaction was developed in a freshly prepared solution of diaminobenzidine (0.5 mg/ml; Sigma) and 0.01% H₂O₂ (30% w/v) for 5 min. The sections were then counterstained with hemalaun, dehydrated, cleared in xylene, and mounted with Entellan (Merck, Darmstadt, Germany).

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³ The abbreviations used are: AR, androgen receptor; PSA, prostate-specific antigen.

LNCaP cells, which contain high levels of the AR protein, were used as positive controls. PC-3 and DU-145 cells served as negative controls. The cells were cultured as described elsewhere (12). Confluent cells were trypsinized, cytospinned, resuspended in PBS, fixed in 1% paraformaldehyde, permeabilized by adding 0.1% of Triton X-100 and stained for AR expression.

Semiquantitative evaluation of AR and PSA immunostaining was performed on the basis of a 4-point scale (–, negative staining; +, <10% of cells positive; ++, 10–50% of cells positive; and +++, >50% of cells positive).

RESULTS

AR Expression in Tumor Metastases. The tissue morphology of the metastatic lesions investigated in our study was well preserved, and staining of good quality could be achieved with the polyclonal antibody PG-21 (Fig. 1). AR positivity was observed exclusively in the nuclei of adenocarcinoma cells in all specimens (Fig. 2) and in the LNCaP cells. PC-3 and DU-145 cells, which were used as a negative control, did not stain for AR. The amount of AR-positive cells was evaluated on the basis of a 4-point scale (Table 1). In eight metastases obtained from seven patients, more than 50% of tumor cells were AR positive. Three patients in this group had been treated with orchiectomy and the synthetic estrogen diethylstilbestrol, whereas three patients had received hydroxyflutamide following orchiectomy. One patient whose tissue specimen contained more than 50% AR-positive cells had been treated with the luteinizing hormone-releasing hormone analogue decapeptyl and the progestagenic antiandrogen cyproterone acetate. In 10 prostatic carcinoma metastases (of 10 patients), the percentage of AR-positive cells was between 10 and 50. Five patients in this group had received hydroxyflutamide following surgical castration, and in one patient, androgen ablation was achieved with decapeptyl and cyproterone acetate. Three patients had undergone orchiectomy alone, and in one patient, no treatment other than irradiation had been performed. In one bone metastasis, in one periosteal metastasis, and in two specimens derived from an epidural space, lesions less than 10% of the tumor cells were found to express the AR. One of the patients from whom these metastases were obtained had undergone orchiectomy alone, one had received flutamide alone, and two had received the nonsteroidal antiandrogen hydroxyflutamide in addition to orchiectomy.

PSA Immunostaining in Metastatic Specimens. In all but one metastasis, PSA could be detected with the help of a polyclonal antibody. This PSA-negative tumor lesion was derived from bone. Interestingly, more than 50% of the tumor cells were AR positive in this sample. All other samples expressed cytoplasmic immunoreactivity for PSA.

DISCUSSION

The most important finding of the present study is that all distant metastases from human prostatic carcinoma contain the immunoreactive AR protein. This may be somewhat surprising because the metastatic samples were derived from progressive prostatic carcinomas, which are thought to acquire an androgen-independent growth pattern during androgen ablation therapy. In view of the fact that AR expression is decreased or absent in highly malignant Dunning tumor sublines and in two human metastatic prostatic cell lines (PC-3 and DU-145), one might expect that metastases from androgen-independent human prostatic carcinoma are devoid of AR (12, 15, 18). The androgen-dependent Shionogi mammary carcinoma, on the other hand, was found to progress to hormone insensitivity in the presence of AR (19). All these cell lines, unlike the *in vivo* tumors analyzed in the present study, represent clonal selections of the original tumor cell populations. For this reason, data obtained from studies on AR expression in tumor cell lines are not really representative of human prostatic cancer metastases.

Immunostaining clearly revealed AR in the cell nuclei of the 22 tumor specimens. The nuclear localization of the AR observed in our study is in agreement with other immunohistochemical studies on AR expression in prostatic carcinoma (4, 5, 20, 21). These studies also correlated AR expression with tumor grade and stage. The majority of primary prostatic carcinomas, even those that do not respond to endocrine therapy, express the AR (4, 5). Heterogeneity in AR expression is more frequently observed in less-differentiated prostatic carcinomas (21). Previous immunohistochemical studies have not provided unequivocal data on the relationship between AR positivity in primary prostatic tumors and response to endocrine therapy. Sadi *et al.* (22) did not find a correlation between the percentage of

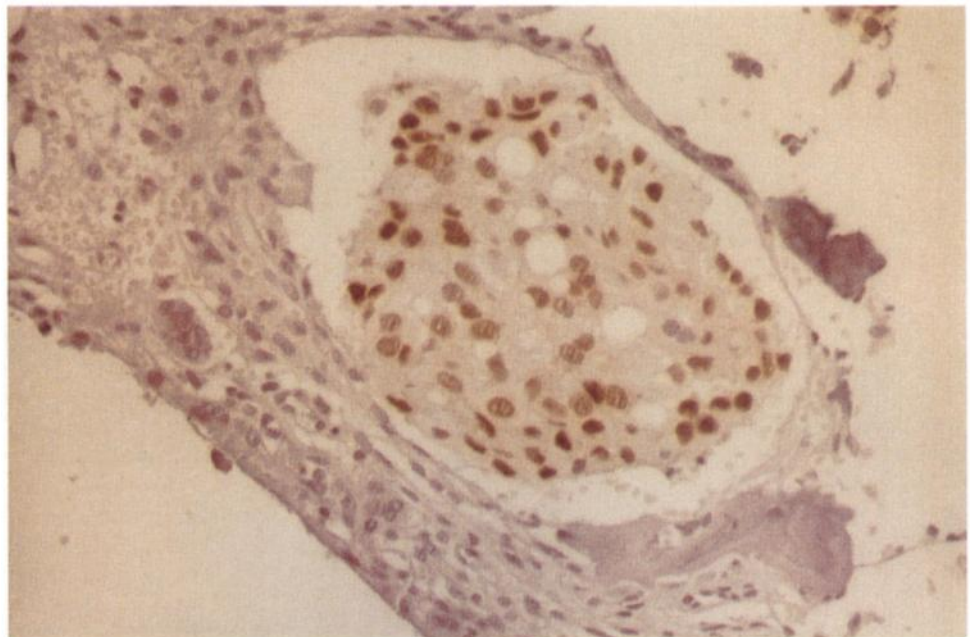
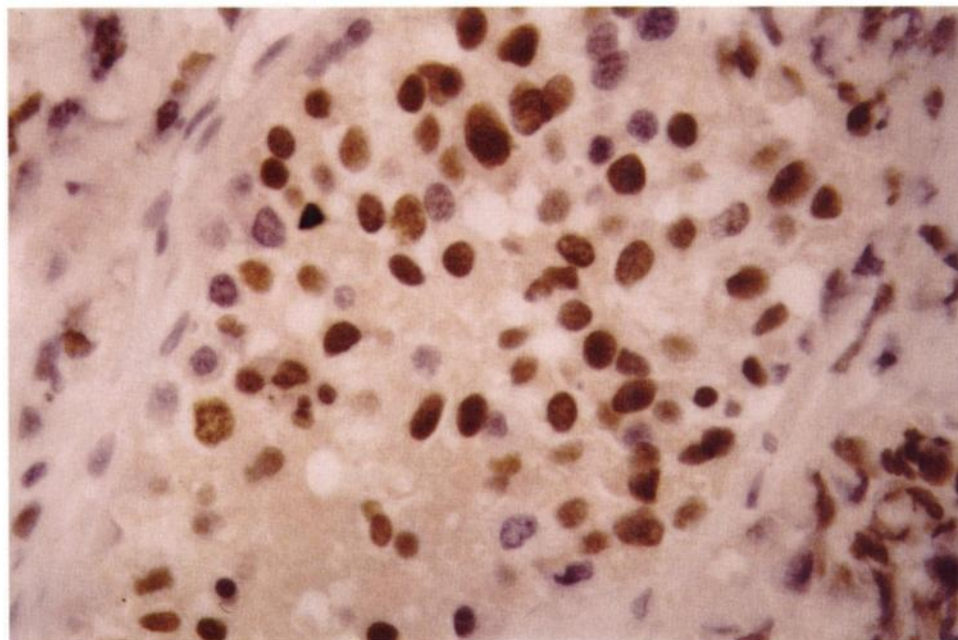


Fig. 1. AR-positive cells in a bone metastasis from prostate cancer ($\times 200$). Paraffin-embedded sections fixed in formalin were stained with the polyclonal antibody PG-21 as described in "Materials and Methods."

Fig. 2. Nuclear expression of AR in metastatic prostatic cancer localized in the epidural space ($\times 400$).



AR-positive malignant cells in the biopsy specimens of 17 patients with stage D prostate cancer and time-to-progression. By contrast, Pertschuk *et al.* (23) reported a significant correlation between the number of AR-positive cells with endocrine response, time-to-progression, and survival. Recent studies that used computer-assisted video image analyses in evaluation of immunohistochemical data have provided evidence that this method has the potential of predicting the outcome in prostate cancer. AR content per cell nucleus is more variable in specimens obtained from patients in whom the time to progression is short (24, 25). The aim of our study, however, was to obtain information about AR expression in the very late stages of prostatic carcinoma; therefore, our data are of no prognostic relevance for the individual patient. Moreover, we did not use this method in the present study because of the differences in processing metastatic

specimens (decalcification of bone samples), which may influence the intensity of immunostaining.

In one poorly differentiated metastasis, no PSA expression was found, despite its strong AR positivity. It has been reported that some prostatic carcinomas, mostly poorly differentiated ones, show no detectable PSA protein levels on immunohistochemical investigation (26). Like all androgen-dependent genes, the PSA gene is regulated via the AR (27). More detailed information about the structure of the AR and the PSA gene in this case may provide some explanation for this phenomenon. One cannot exclude the existence of a molecular defect that generates AR whose function is impaired. In addition, the PSA gene can be inactivated or lost. Bovenberg *et al.* (28) have reported no significant difference between prostatic primary tumors and their metastases as to PSA expression. In their study, all nine metastases analyzed stained for PSA (28).

In our study, none of specimens were negative for both the AR and the PSA. This is in contrast to the androgen-insensitive tumor cell lines PC-3 and DU-145, which are negative for the AR as detected by immunoblotting, and do not express the PSA protein either, although the genes encoding both AR and PSA can be detected (13, 15). Thus, our findings indicate that these two cell lines are not representative of distant metastases from prostatic carcinoma.

In-depth knowledge of the mechanisms governing AR expression and function in an androgen-depleted environment is essential for developing a more rational endocrine therapy for metastatic prostatic carcinoma. To settle the issue whether the AR is indeed involved in prostatic cancer progression, several molecular mechanisms have to be considered. They may involve up-regulation of AR expression following androgen deprivation, structural alterations, or nonsteroidal activation of the receptor. These mechanisms have been studied in various experimental systems. Quarby *et al.* (29) observed an increase in the AR mRNA level in the LNCaP cells maintained in serum-free medium. Kokontis *et al.* (30) have recently developed a new *in vitro* model that mimics the situation in human prostatic carcinoma following androgen ablation. The main advantage of this model is that it reflects alterations in AR expression and function after long-term androgen withdrawal. The LNCaP cell subline, which was established after about 60 passages in an androgen-depleted medium, displayed a 2.5-fold increase in the AR mRNA level and a 15-fold

Table 1 AR and PSA expression in prostatic carcinoma metastases

Patient no.	Tumor grade	Localization of metastasis	Expression of		Therapy ^b
			AR ^a	PSA ^a	
1	III	Bone (C)	+++	+	O+DES
2	II	Bone (ilium)	+	+	O+F
3	III	Bone (femur)	+++	-	O+DES
4	III	Epidural space	+++	+	O+DES
5	II	Bone (VC)	++	++	O+F
5	II	Bone (humerus)	+++	++	O+F
6	II	Bone (VC)	++	+	I
7	III	Bone (VC)	+++	+	DCP+CPA
8	II	Bone (ilium)	++	+++	O
9	II	Bone (VC)	++	+++	O+F
10	III	Bone (VC)	++	+	O+F
10	III	Bone (humerus)	+++	+	O+F
11	III	Bone (VC)	+++	+++	O+F
11	III	Bone (humerus)	+++	++	O+F
12	III	Bone (VC)	++	+++	O
13	II	Bone (VC)	++	+++	O
14	III	Periosteum (C)	+	+	O
15	III	Bone (VC)	++	++	O+F
15	III	Epidural space	+	+	O+F
16	II	Bone (VC)	++	+++	DCP+CPA
17	III	Epidural space	+	+	F
18	III	Bone (VC)	++	++	O+F

^a -, negative staining; +, <10% cells positive; ++, 10–50% cells positive; +++, >50% cells positive.

^b O, orchietomy; DES, diethylstilbestrol; F, hydroxyflutamide; I, irradiation; DCP, LHRH analogue decapeptyl; CPA, cyproterone acetate; C, cranium; VC, vertebral column.

increase in the AR protein. These changes are associated with a 20-fold increase in AR activity and enhanced biphasic proliferative response of LNCaP cells to low doses of the synthetic androgen methyltrienolone (R 1881; Ref. 30). Possibly, some prostatic cancers adapt to the androgen-depleted environment by increasing the level of the AR protein and its activity. This would imply that, even in orchiectomized patients, the small amounts of androgen supplied by the adrenals are sufficient to activate the AR. By contrast, de Winter *et al.* (31) reported no significant changes in AR mRNA in the human transplantable prostatic carcinoma cell line PC-82 within 5 days after androgen withdrawal. Nevertheless, the AR protein in this cell line, which had not been detectable on immunohistochemistry within 5 days after castration, was restored after androgen substitution (31).

Currently, very little is known about the structure of the AR in prostatic carcinoma metastases (9, 32). Provided, however, point mutations were to occur more frequently in metastases than in primary tumors, this would constitute a potential mechanism enabling tumor cells to activate the AR in an androgen-depleted environment. For example, the mutant AR with a Val→Met exchange in the region encoded by exon D of the AR gene is more efficiently activated than the wild-type AR by the adrenal androgens, dehydroepiandrosterone and androstenedione; the dihydrotestosterone metabolites, androsterone and androstandiol; and progesterone (7, 33). The mutant AR detected in the LNCaP cell line that shows a Thr→Ala exchange in the region encoded by exon H of the AR gene has an increased binding affinity for estrogen, progestagenic steroids, and the nonsteroidal antiandrogens hydroxyflutamide and nilutinamide (34). In transactivation studies, these steroids and antihormones were found to be more effective with this mutant receptor than with the wild-type AR (35). Some mutant ARs that are present in metastases may have similar properties as the LNCaP AR. This is suggested by the clinical observation that in some patients, discontinuation of flutamide during androgen ablation therapy leads to a decline in PSA and a temporary improvement in the patient's condition (36). Thus, activation of mutant ARs in prostatic tumor metastases by antiandrogens may be considered as one possible mechanism in tumor progression.

There is increasing evidence that substances other than androgen have a role in the stimulation and suppression of AR-mediated gene transcription (37–39). Polypeptide growth factors such as insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor stimulate AR-dependent reporter gene activity, depending on cell and promoter context (37). As has been demonstrated in studies on CV-1 cells cotransfected with a reporter gene and an AR expression vector, the second messenger cyclic AMP is able to enhance androgen effects (38). Retinoic acid has been shown recently to abolish induction of AR-mediated chloramphenicol acetyltransferase gene activity (39). These signaling pathways, which may be particularly important for AR function in metastases from prostatic carcinoma, warrant further investigation.

In conclusion, this study provides the first evidence that distant metastases obtained from patients with prostatic carcinoma who have undergone various kinds of endocrine therapy do not lack the AR. Together with the results of recent studies on alterations in the transmission of androgen signals in advanced prostatic carcinoma, our findings support the hypothesis that the AR is involved in prostatic tumor progression.

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