Malignant Transformation of Human Prostatic Epithelium Is Associated with the Loss of Androgen Receptor Immunoreactivity in the Surrounding Stroma

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ABSTRACT
The cellular pathways involved in the pathogenesis of hormone resistance remain unclear. Studies evaluating the role of changes in human androgen receptor (hAR) expression in the progression of prostatic tumors have been inconclusive. Androgenic influence over prostatic growth is mediated via the regulation of interactions between stromal and epithelial cells. We hypothesized that neoplastic transformation of the prostate would be associated with alterations in hAR expression in the adjacent stroma. Using immunohistochemical techniques, we determined hAR positivity in the epithelium and adjacent stroma of sections from 17 benign and 39 malignant prostatic glands. We found that whereas the expression of the receptor decreased in both cellular compartments as the tissues dedifferentiated, the depletion was more pronounced in the stromal nuclei ($P < 0.0001$). However, in sections from both untreated and hormone-resistant prostate cancer tissues, although heterogeneity of hAR expression in malignant epithelia was increased, there appeared to be a unique field effect around the cancerous prostate glands that resulted in a decreased expression of the receptor in the adjacent benign glands and its total loss in the surrounding stroma. The loss of hAR in the stroma adjacent to malignant prostatic epithelium may play an important role in prostate cancer progression. Furthermore, the similarity of the lack of stromal hAR expression in newly diagnosed and hormone-resistant prostate cancer ($P = 0.85$) may be an indication that the mechanisms responsible for the acquisition of hormone independence are established early in the malignant transformation process.

INTRODUCTION
Androgenic control over the normal growth and differentiation of the prostate gland is transmitted via hARs that are expressed by the nuclei of both stromal and epithelial cells (1). The mechanisms responsible for the initiation and propagation of prostatic carcinogenesis and the development of hormone resistance in CAP are still unknown, but abnormalities in the activity of these steroid receptors are thought to play an important part in these changes in the prostatic cell phenotype. As a result, evaluation of hAR expression in normal and abnormal prostatic tissues has been a major focus of efforts to unravel the mechanisms responsible for malignant transformation and hormone-independent growth of the organ.

In the earliest studies, hAR content of prostatic tissues was assessed using biochemical assays, but these studies were considered nonspecific because the substrates analyzed were a heterogeneous mixture of stromal elements and benign and malignant epithelial cells (2). The recent development of anti-hAR-specific antibodies has made it possible to determine hAR expression in the individual glands and cellular compartments within prostatic tissue sections. Studies using this newer methodology have concentrated mainly on the tumor cells and have reported conflicting findings. Whereas initial reports suggested that hAR protein expression in epithelial nuclei was inversely proportional to the histological grade of the tissues (3, 4), more recent studies have detected high expression and increased heterogeneity of the receptor in advanced cancers (5, 6). These later reports are supported by scrutiny of prostate cancer tissues using reverse transcriptase techniques, which has revealed amplification of the hAR gene in hormone-refractory tumors (7, 8).

hAR transmission of hormonal control of prostatic growth occurs via the regulation of the expression of the peptide growth factors and receptors involved in the stromal-epithelial interactions that mediate the influence of androgens on the gland (9, 10). Proliferation and differentiation of prostatic epithelium is initiated by the binding of epithelial cell membrane receptors by growth factors expressed by stromal cells (11). This intercellular relationship indicates that prostatic stroma plays an important intermediary role in the transmission of androgen-induced stim-

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1 This work is dedicated to Prof. T. F. Solanke (contemporary). These results were presented in part at the British Association of Urological Surgeons Meeting in Bournemouth, United Kingdom, June 21–24, 1997 and at the Schilling Research Conference, Hormones and Cancer in Santa Cruz, California, September 18–21, 1997.

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3 The abbreviations used are: hAR, human androgen receptor; CAP, carcinoma of the prostate; BPH, benign prostatic hyperplasia; HG, high grade; PIN, prostatic intraepithelial neoplasia.
buffered 3 was applied, followed by a freshly prepared chromogen mix of antimouse antibody, and then the streptavidin-biotin complex anti-hAR. The sections were incubated with a goat secondary antibodyated overnight at 4°C with a prediluted solution of mouse IgG. The sections were then blocked with normal goat serum and incubated in archival sections of benign and malignant prostatic tissue using immunohistochemical methods.

**MATERIALS AND METHODS**

**Archival Material.** Sections from archival specimens of transurethral and radical retropubic prostatectomies from 17 patients with BPH and 39 age-matched patients with CAP were supplied by the Pathology Department of Leicester General Hospital. Thirty-two of the malignant cases were from newly diagnosed patients who had not been treated with hormonal ablation, whereas 7 cases were from patients with hormone-resistant CAP. The tissues had been fixed in 10% buffered formalin and embedded in paraffin. Representative sections from each block of archival tissue were stained with H&E for routine histological evaluation, and suitable blocks for examination were selected by an experienced uropathologist (E. H. M.). The malignant prostatic tumors were graded using the Gleason score (12) and classified as well differentiated (G1, Gleason score 2–4), moderately differentiated (G2, Gleason score 5), or poorly differentiated (G3, Gleason score 8–10).

**hAR Immunostaining.** Using a commercially available monoclonal anti-hAR antibody (NCL-2F-AR; Novocastra), hAR immunostaining was done as described previously (13). Briefly, the archival sections were deparaffinized and rehydrated in xylene and graded alcohols. They were equilibrated in TBS, and then intrinsic peroxidase activity was quenched with hydrogen peroxide. Exposure of hAR epitopes was achieved by microwaving the sections in 10 mM citrate buffer (pH 6.0). All sections were then blocked with normal goat serum and incubated overnight at 4°C with a prediluted solution of mouse anti-hAR. The sections were incubated with a goat secondary antimouse antibody, and then the streptavidin-biotin complex was applied, followed by a freshly prepared chromogen mix of buffered 3',3'-diaminobenzidine tetrahydrochloride (with heavy metal enhancement to give a black-positive reaction). The sections were dehydrated, cleared, and mounted with XAM neutral mounting medium (BDH Poole) without counterstaining. All staining runs included positive controls (CAP sections known to be hAR positive) and negative controls (sections of transitional bladder carcinoma incubated with anti-hAR and prostatic sections incubated with antimouse IgG instead of the mouse anti-hAR). As we described previously (14), all sections were stained within 10 days of preparation to exclude the loss of immunoreactivity due to storage-induced antigen degradation.

**Evaluation of Immunochemistry.** hAR-positive immunoreaction was defined as black nuclear staining that was present in test sections stained with anti-hAR antibody but absent in negative controls. False positivity due to formalin pigments was excluded by viewing the slides with polarized light. Also, areas of inflammation, infarction, and tissue autolysis were not assessed. The hAR score was independently determined by two separate observers (E. O. O-O. and E. H. M.) by counting 400 luminal epithelial and stromal cells in normal, benign hyperplastic, and malignant glands and their surrounding stroma at ×400 magnification from selected areas of the sections (see below). The number of cells with a positive nuclear reaction was then expressed as a percentage (15). The final score was the average of the scores awarded by the two observers. In cases where the scores given differed by a margin of >5, the sections were jointly reexamined, and a score was agreed upon. The final scores were classified as follows: 0, negative; 1–33%, weak expression; 34–66%, moderate expression; >66%, high/strong expression.

The level of hAR expression was first determined in areas of histologically homogeneous BPH and CAP. The effect of neoplastic epithelium on the surrounding stroma was also determined by assessing hAR positivity in areas of normal/atrophic and nodular (stromal) hyperplasia in BPH sections and in adjacent and distant benign (normal/atrophic and benign hyperplastic) glands and their surrounding stroma in CAP sections. Adjacent glands were defined as those within the same high-powered field, whereas distant glands were defined as being more than two high-powered fields from a malignant gland (16).

**Statistical Methods.** The results were reported as proportions within histological groups. The unpaired t test was used to compare the mean hAR scores in the different histological groups when the distributions were approximately normal, and the Mann-Whitney test or Fisher’s exact test was used otherwise. Comparison of the mean hAR scores in the different histological areas within the same sections was done using the paired t test or McNemar’s test as appropriate. The univariate association between stromal and epithelial hAR expression and hormone resistance was examined using Spearman’s correlation coefficient. All tests were two-sided and performed at the 0.05 level of significance.

**RESULTS**

The specificity and sensitivity of the anti-hAR antibody used to recognize its antigen were confirmed by the absence of staining in negative controls and the strong reaction of the positive control sections (Fig. 1, A–C).

A detailed account of immunostaining patterns observed in BPH and prostate cancer sections is summarized in Table 1.

**Androgen Receptor Immunostaining in BPH Sections**

In benign prostatic sections, hAR expression was seen in both epithelial and stromal nuclei. However, the immunoreaction in both normal/atrophic and BPH glands within the sections was almost completely confined to the luminal epithelium because very few basal cells were hAR positive. The intensity of hAR staining was more uniform in normal/atrophic glands within these sections than in the adjacent BPH glands that stained heterogeneously.
Fig. 1 Confirmation of anti-hAR antibody (NCL-2F-AR) specificity. A, lack of hAR expression in a section of transitional cell carcinoma stained with anti-hAR antibody (negative control; ×400). B, CAP section incubated with antimouse immunoglobulin antibody showing no staining (negative control; ×250). C, strong anti-hAR staining in a malignant gland in a known hAR-positive CAP section (positive control; ×400).
A detailed description of the pattern of hAR positivity in the epithelium and stroma of BPH and CaP sections is shown. This shows a gradual decrease in immunoreactivity as the tissues dedifferentiate, with hAR expression in the stroma being significantly lower than that of the adjacent epithelium within each histological grade.

<table>
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<tr>
<th>Epithelium vs. stroma</th>
<th>Epithelium Mean</th>
<th>Surrounding stroma Mean</th>
<th>P</th>
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<td>&lt;0.0001</td>
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<tr>
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<td>(total no.)</td>
<td>(total no.)</td>
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<td>51 (13)</td>
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<td></td>
<td>17 (100)</td>
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<td>0 (0)</td>
<td>16/17 (94)</td>
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<td>51 (13)</td>
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<td>(SD)</td>
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<td>(total no.)</td>
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| Androgen Receptor Expression in CAP Sections

hAR was also highly expressed in epithelial nuclei in all BPH sections (Fig. 2B). However, hAR expression was significantly higher in normal/atrophic glands than in BPH glands within the same sections (P = 0.002).

Stroma Surrounding CAP and HG PIN Glands. hAR was absent in the nuclei of stromal cells surrounding malignant epithelia in most sections from CAP glands (Fig. 3, A and B). Although the lack of stromal expression of hAR was more apparent in the higher-grade tumors, the difference between the mean scores of adjacent histological grades was not statistically significant (P = 0.28 and 0.69, respectively). Similar to CAP glands, the stroma around HG PIN glands in the majority of sections lacked hAR expression (HG PIN versus CAP, P = 0.61).

Stroma

Heterogeneous hAR expression was seen in the nuclei of stromal cells in all BPH sections. However, hAR immunostaining was highest in areas of nodular stromal hyperplasia (Fig. 2, A and B) and decreased progressively in stroma surrounding normal/atrophic and BPH glands (P = 0.02 and 0.0004, respectively).

Epithelial Tissue

hAR was also highly expressed in epithelial nuclei in all BPH sections (Fig. 2B). However, hAR expression was significantly higher in normal/atrophic glands than in BPH glands within the same sections (P = 0.002).

Androgen Receptor Expression in CAP Sections

hAR staining in both epithelial and stromal nuclei in CAP sections was more heterogeneous than that in BPH sections (Fig. 3, A and B). Whereas all histologically benign epithelium within our sections expressed the receptor, 5 of 39 CAP glands had foci of malignancy that were hAR negative (Fig. 3C). One of these glands was a well-differentiated (G1) cancer, whereas two each were G2 and G3 tumors.

Stroma

Stroma Surrounding Adjacent and Distant Benign Glands in CAP Sections. Benign glands adjacent to CAP glands were also surrounded by hAR-depleted stroma, with a gradual reappearance of the receptor in the stroma around the more distant benign epithelium (Fig. 3, A and D). This gradual change was more easily seen in whole mount sections of radical prostatectomy specimens. The weak hAR expression in the stroma surrounding the adjacent benign glands was slightly higher than but not significantly different from that of stroma within homogeneous areas of malignancy within the same sections (P = 0.30). However, it was significantly less than the expression of the receptor by the stroma around distant benign glands of the same section (P = 0.006). In addition, hAR expression in the stroma surrounding the peripheral benign glands in CAP sections was statistically similar to that of the stroma around benign glands in BPH sections (P = 0.40; Figs. 2B and 3D).

Glandular Tissue

CAP Epithelium. Despite the increased heterogeneity of hAR staining in malignant prostatic epithelium, the mean hAR expression in CAP glands was significantly less than that in normal/atrophic and BPH glands in BPH sections (P < 0.0001). Although the mean hAR expression was higher in the advanced
cancers, there was no significant difference between the mean scores of adjacent histological grades ($P = 0.39$ and $0.62$, respectively).

**HG PIN Epithelium.** Mean hAR expression in HG PIN glands was intermediate between that of benign and malignant glands. However, whereas the HG PIN hAR mean score was significantly less than that of the BPH glands ($P = 0.005$), it was statistically similar to that of CAP glands ($P = 0.5$).

**Adjacent and Distant Benign Epithelium in CAP Sections.** hAR expression was lower in adjacent histologically benign glands than it was in distant histologically benign glands in our malignant sections, but the difference was not statistically significant ($P = 0.10$; Fig. 3, A and D). However, hAR immunopositivity was significantly less in these benign glands than in CAP or HG PIN glands within the same sections ($P = 0.012$ and 0.007, respectively; Fig. 3A). Furthermore, the mean hAR score of benign glands in CAP sections was markedly lower than that of benign glands in BPH sections ($P = 0.0001$).

**Stromal and Epithelial hAR versus Hormone Resistance in CAP**

Comparison of hAR positivity in untreated and hormone-refractory CAP glands showed similar expression of the receptor in both the epithelium and the stroma of both types of glands.
Subsequent examination of the Spearman correlation coefficients of hormone insensitivity with hAR expression in the epithelial and stromal compartments by univariate analysis also revealed no significant association between the indices ($P = 0.59$ and 0.15, respectively).

**DISCUSSION**

The uncertainty surrounding the relationship between hAR expression, advancing histological grade, and hormone resistance in prostatic tumors encouraged us to undertake this project. Although the newer and more specific immunohistochemical and genetic tests have gone some way in resolving the nonspecificity of the earlier biochemical assays, the role of the receptor in the progression of CAP remains unclear. Until now, however, these recent studies have focused essentially on hAR expression in epithelial nuclei in the prostatic specimens examined, presumably because these are the primary cells involved in neoplastic transformation. We were of the opinion that because androgen-regulated interactions between stromal and epithelial cells are mandatory for both normal and abnormal growth of the prostate, a determination of the levels of hAR expression in only one cellular compartment of the organ represented a possible flaw in the results obtained and thus in the interpretation of the same. We reasoned that the evaluation of the differential expression of the receptor in both stromal and epithelial nuclei in benign and malignant prostatic neoplasms would yield vital information that could improve our understanding of the mechanisms responsible for the progression of CAP.

In general, nuclear hAR expression in the epithelium of our specimens decreased as the tissues became increasingly neoplastic, whereas the heterogeneity of the staining increased. Interestingly, hAR positivity in benign glands in our CAP sections was significantly lower than that seen in the epithelium in BPH sections, but the significance of this previously unreported finding is presently unknown. Within the cancers however, the mean expression of the receptor was increased in the poorly differentiated (G3) tumors. The lower mean hAR score recorded in our moderately well-differentiated G2 tumors as compared to the well-differentiated cancers is probably due in part to the disproportionately higher number of androgen receptor-negative tumors within the group [20% (G2) versus 10% (G1) and 11% (G3), respectively]. However, we found no significant correlation between the expression of the receptor by epithelial nuclei and hormone resistance in our specimens. In addition, hAR

![Fig. 3](image_url) Anti-hAR staining in primary human prostate cancer. A, area of heterogeneous staining of malignant epithelium (arrows) in the midst of hAR-negative stroma in a whole mount CAP section ($\times 250$). Note the weaker hAR staining in the adjacent phenotypically benign (normal/atrophic) and benign hyperplastic (b) glands that are also surrounded by hAR-depleted stroma, suggesting the presence of a field effect around CAP glands. B, malignant prostatic glands surrounded by hAR-positive stroma ($\times 250$). C, foci of mainly hAR-negative CAP glands (arrows; $\times 250$). Note that the surrounding neoplastic cells show heterogeneous immunoreactivity for hAR, whereas the stroma lack expression of the receptor. D, area of distant benign glands in the whole mount CAP section shown in A with heterogeneous hAR staining in the epithelium and stroma similar to that observed in the BPH sections ($\times 250$).
expression was significantly higher in the epithelium than in the adjacent stroma, irrespective of glandular histotype. Because these findings are in keeping with those of previous studies (5, 6, 15), the rest of this discussion will focus on the varied expression of the receptor in the stroma of our specimens.

We believe that this is the first definitive report of a more pronounced decrease in expression of hAR in stromal cells (as compared to the epithelium) as prostatic tissues dedifferentiate. We found only one report in the literature that noted the absence of the receptor in the stroma of a minority of sections from untreated CAP glands, and although the proportion increased after hormonal ablation, the difference was not significant (17). This is in contrast to comments from other studies which, although directed at testing other hypotheses, reported some heterogeneous staining of stromal cells irrespective of the histology of the adjacent epithelium (3, 15, 18). The validity of our results is supported by the demonstrable specificity of the antibody used and the similarity of the pattern of hAR expression in the epithelial compartment of our specimens with those of previous studies. In addition, technical artifacts were excluded by the presence of heterogeneous staining in the adjacent malignant epithelium.

Several reasons may be responsible for the novelty of our findings, and these would include: (a) the specific aim, ab initio, to determine the expression of hAR in the stromal and epithelial cells in our sections; (b) the use of archival sections of BPH and CAP in which the preservation of tissue architecture made it possible to evaluate staining in the two cellular compartments; (c) the use of a monoclonal anti-hAR antibody that recognizes antigens on archival material; and (d) the evaluation of the effect of proximity to a neoplastic epithelium on hAR expression in the stroma in both BPH and CAP sections.

We are presently unable to explain the absence of hAR positivity in the stroma around neoplastic epithelium in our sections, but one possible cause is the presence of mutant receptors that are not recognized by the antibody used. However, although there have been several reports of aberrant receptors in cancerous prostate tissues (19–21), in general, they are considered to be uncommon in primary prostatic tumors. The finding may also be due to abnormal interactions between

the stromal and epithelial elements of the malignant prostate gland. Previous experiments have shown that mitotic human epithelium is capable of inducing neoplastic changes in the adjacent stroma (22, 23), although the responsible mechanisms are unknown. The graded reduction of hAR expression in the stroma in the vicinity of altered prostatic glands, culminating in the total absence of the receptor in the stroma surrounding fully malignant cells, may therefore be due to the effect of as yet undetermined tumorigenic signals from transformed epithelial cells. The similarity of the depletion of hAR in the stroma surrounding both cancerous and adjacent benign glands in our CAP sections, whereas the stroma surrounding distant benign epithelium in the same sections was hAR positive may also be important, because it suggests that the expression of the receptor in the stromal nuclei may be determined primarily by the proximity of a malignant focus, and not the histology of the adjacent gland. This in vivo evidence is in support of previous suggestions from human and animal studies that local extension of primary and metastatic cancers may rely, at least in part, on the mutagenic field effect of the altered surrounding stroma (24–26). When considered with the detection of a decreased hAR expression in adjacent benign glands in CAP sections as compared to the glands in BPH sections, it seems possible that there is a similar oncogenic field effect in the epithelial compartment as well. However, further research is required to confirm these findings.

There was no significant association between the loss of stromal hAR expression and hormone resistance in our specimens, and this is probably due to the absence of expression of the receptor in the stroma of untreated and hormone-refractory prostatic glands alike. However, the loss or mutation of hormone receptors is thought to be one of the pathways by which hormone-sensitive tumors acquire hormone resistance. The possibility that changes in stromal hAR expression (such as those demonstrated in this study) in prostatic neoplasms could be significant in this regard is strengthened by the importance of the intermediary role of this cell type in the mediation of androgenic control of the gland (24). Furthermore, because hAR is required for the expression of peptide growth factors, it is possible that with the loss of this receptor, stromal cells cease to express these proteins early in the malignant transformation of the prostate. The survival of malignant cells would then rely on their ability to switch to hormone-independent cellular pathways (including those responsible for autonomous epithelial proliferation such as we have recently described4) sooner rather than later following transformation. In contrast, hAR gene amplification in epithelial cells has been detected only in tumors that have gradually become hormone resistant, i.e., later in the natural history of the disease (6, 7). Thus, it seems possible that those alterations in the molecular biology of the stromal compartment of the prostate involved in the progression to hormone-refractory disease occur during or soon after malignant transformation of the epithelium, perhaps preceding the genetic

### Table 2  hAR expression in newly diagnosed and hormone-resistant prostate cancer

A comparison of mean hAR scores in the epithelium and stroma of sections from newly diagnosed and hormone-refractory CaP cases confirms the similarity of differential hAR expression in both stages of the disease. This suggests that the loss of hAR expression in stromal nuclei is an early rather than late event in malignant prostatic disease.

<table>
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<tr>
<th>Hormone status (total no.)</th>
<th>Epithelium Mean score (%) (SD)</th>
<th>Surrounding stroma Mean score (%) (SD)</th>
<th>Epithelium vs. stroma</th>
<th>P</th>
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*ND CaP, newly diagnosed prostate cancer; HR CaP, hormone-resistant prostate cancer.*

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changes in the epithelial cells that are thought to contribute to the same phenotype.

We have found that hAR expression decreased in human prostatic epithelial and stromal cells in proportion to the degree of differentiation of the tissues, with the decline being more pronounced in the stroma. In view of the hypothesis that mutagenic epithelia induce changes in the surrounding stroma, this report of a loss of expression of the receptor in the stroma surrounding CAP glands may be the first in vivo evidence of the possible mechanisms by which tumor stroma is formed. This abnormal stroma may then play an important role in both the progression of malignant transformation within the gland and the transmission of the potential for hormone-independent growth. We believe that the similarity in lack of hAR expression in the stroma in untreated and hormone-resistant CAP cases suggests that the cellular events responsible for the development of hormone resistance may occur during or shortly after carcinogenesis.

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REFERENCES


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