

Amplification of the androgen receptor may not explain the development of androgen-independent prostate cancer

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Objective To examine the role of androgen receptor (AR) gene amplification and aneusomy of the X chromosome in the development of antiandrogen-resistant prostate cancer.

Patients and methods Twenty patients with prostate cancer resistant to androgen-deprivation therapy were selected for study. The records of patients with tumours before and after antiandrogen therapy, and with a full clinical follow-up, were retrieved. AR gene amplification and X chromosome copy number were assessed by fluorescence *in situ* hybridization using a labelled probe at locus Xq11–13 for the AR gene and a labelled α -satellite probe for the X chromosome. At least 20 nuclei were scored over three tumour areas by two independent observers.

Results Aneusomy of the X chromosome was reported respectively in seven (35%) and 11 (55%) tumours before and after hormone relapse, the AR gene copy number was increased in seven (35%) and 13 (65%),

respectively, and AR gene amplification was detected in one (5%) and three (15%), respectively. Neither increased AR copy number nor AR amplification in primary tumours precluded a biological response to androgen-deprivation therapy.

Conclusion The rate of AR gene amplification is too low to be solely responsible for the development of antiandrogen-resistant prostate cancer. Also, the presence of amplified AR and cells aneusomic for the X chromosome in primary tumours that respond to androgen-deprivation therapy suggests that an increase in AR gene copy number does not prevent a tumour from responding to this therapy. Therefore other mechanisms which could cause hormone-refractory prostate cancer must be investigated before it is understood why so many patients relapse with this disease.

Keywords prostate cancer, androgen receptor, X chromosome, fluorescence *in situ* hybridization

Introduction

The incidence of prostate cancer is higher in men from the western world than any other cancer and is the second most frequent cause of male cancer-related deaths [1–3]. As androgens regulate normal growth and differentiation of the prostate gland, it is not surprising that prostate cancer growth is stimulated by androgens [4]. The effects of androgens on prostate tissue can be inhibited by androgen receptor (AR) antagonists (anti-androgens) [5]; 70–80% of men with prostate cancer treated with antiandrogens respond favourably [6]. However, this effect is transient [7], with most patients eventually developing androgen-resistant disease [8].

Several theories, e.g. mutation of the AR [7], clonal expansion of tumour cells hypersensitive to androgens [9] and increased expression of AR [4], have been proposed as a possible explanation as to why prostate cancer develops resistance to antiandrogens or

androgen-deprivation therapy. Koivisto *et al.* [10] postulated that amplification of the AR gene plays a key role in progression to refractory prostate cancer [9,10]. They reported that six of 10 recurrent prostate tumours resistant to antiandrogens and androgen-deprivation therapy were polysomic for the X chromosome and that three had amplification of the AR gene [10]. They hypothesized that the combination of these factors caused the development of prostate cancer resistant to current first-line therapy, i.e. an increase in AR protein levels would result in proliferative advantages during antiandrogen therapy [10]. Further studies with more patients confirmed that polysomy of X chromosome ranges is detectable in 42–60% of relapsed tumours from patients with prostate cancer, and amplification of the AR gene has been reported in 20–30% of recurrent tumours from the same patient cohorts [9–12].

The largest study to date (339 samples) reported no AR amplifications in any of the 223 primary tumours investigated [12]. However, there is still no clear

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investigation into AR gene amplification before hormonal therapy and after the development of antiandrogen resistance (after hormonal therapy) using paired tumours from the same patient. In the present pilot study we investigated AR gene amplification and X chromosome status in 42 tumours from 20 patients, all with paired primary and relapsed (after androgen-deprivation therapy) tumours from the same patient.

Patients and methods

Twenty patients (mean age at diagnosis 71.1 years, range 55–83) were retrospectively selected for analysis; ethical approval was obtained from the local research and ethical committee for the use of their tissue in the study. All patients received conventional androgen-deprivation therapy (orchidectomy or antiandrogens). Patients were selected for analysis if they initially responded to treatment (the response being defined by the PSA level decreasing by at least half) but subsequently relapsed. PSA values and a full clinical follow-up were available for each patient. Patients were classed as having hormone-refractory cancer when sustained rising PSA levels were recorded and they were selected for study if a tumour sample was available after hormone relapse. The initial tumour sample was either from TURP or TRUS-guided biopsy, but the relapsed tumour sample was always from TURP, which was used to treat clinical symptoms.

Sections (5 µm) were cut from archival formalin-fixed, paraffin-embedded tissue and placed on aminopropyl triethoxysilane-treated slides. The slides were pretreated on a VP2000 robotic slide processor (Vysis Ltd, Surrey, UK). This involved dewaxing and rehydration, pretreatment with 8% sodium thiosulphate at 80°C for 30 min and digestion with 0.05% pepsin at 37°C for 26 min. Tissue sections were subsequently post-fixed in 10% formalin for 10 min and dehydrated through increasing concentrations of ethanol.

Slides were denatured at 72°C for 2 min; dual-labelling hybridization with CEP buffer (Vysis) containing X chromosome α -satellite probe (Spectrum Green[™] labelled CEP X, Vysis) and AR probe (Spectrum Orange[™] labelled probe locus Xq11–13, Vysis) was undertaken at 37°C overnight on a Misha unit (Shandon, Runcorn, UK). Slides were then washed in 0.4 × saline-citrate buffer, 0.3% NP-40 at 72°C for 2 min, allowed to air dry and mounted in 0.5 µg/mL 4,6-diamidino-2 phenylindole-2 hydrochloride in Vectashield antifade (Vetrolabs, Peterborough, UK). Signals were visualized using a microscope with a 100 W mercury lamp, with a triple band-pass filter block specific for the excitation and emission wavelengths of the Spectrum Green and Spectrum Orange fluors (Vysis).

Serially sectioned haematoxylin and eosin-stained tissue sections were first examined microscopically to locate the tumour areas. Sections stained for fluorescence *in situ* hybridization were then scanned at ×100 magnification to locate the tumour areas. Twenty non-overlapping nuclei per section were evaluated from three different areas by two independent observers, using a ×100 objective. Signals per nucleus for X chromosome (green) and AR (orange) were counted on a cell-by-cell basis and the results recorded manually; cells with no signals for either X or AR were ignored. The mean chromosomal copy number for the X chromosome was calculated by totalling the number of green signals (X probe) counted in a specific area and dividing by the number of nuclei assessed. Similarly, the mean gene copy number of the AR was calculated by totalling the number of orange signals (AR probe) counted in a specific area and dividing this by the number of nuclei assessed. Analysis of copy number in thin tissue sections can be affected by nuclear truncation [12,13] and we excluded nuclei with missing signals to ensure that the gene:chromosome ratio was more accurately represented. Therefore, the normal range for X chromosome and AR copy number were identified using the mean chromosomal copy number from 14 BPH (as a genetically stable control) samples ± 3 SD ($\approx 99\%$ CI). Using this approach the normal range for the AR and chromosome X were 0.91–1.30 and 0.97–1.23, respectively. The AR:X chromosome ratio in 14 BPH samples was 0.93–1.07 (1.04 ± 3 SD). Amplification was defined as an AR:X ratio of >1.5 [9]. If tumours were heterogeneous the area with most abnormalities was used in the final analysis.

Fisher's exact test was used to compare the level of X chromosome copy number, AR copy number and AR gene amplification between primary tumours and those after hormone deprivation therapy.

Results

Of the 20 patients, two had bone metastases at the time of initial hormone therapy; 42 tumours were analysed, including 20 primary tumours and 20 tumours after relapse; in addition, two patients underwent TURP during their PSA response and these samples were also analysed. Of the 20 primary tumours, 11 (55%) had a Gleason sum of ≤ 6 , four (20%) a sum of 7 and three (15%) a sum of ≥ 8 . The Gleason score was unavailable for two primary tumours. The Gleason sum for the relapsed tumour was always the same or higher than that of the primary tumour; two patients had a Gleason sum of 7 (10%) in the relapsed tumour and 18 (90%) had a Gleason score of ≥ 8 . One tumour (after hormone relapse) showed heterogeneity (2.3%). In six cases no abnormalities of either the X chromosome or AR copy

number were detected in either the biopsies taken before or after hormone relapse.

Seven (35%) of the primary tumours and 11 (55%) relapsed tumours were polysomic for the X chromosome (Table 1). There was no significant difference in the number of tumours with aneusomy X before and after hormone relapse ($P=0.2$). In two cases the primary tumours were aneusomic for chromosome X and normosomic in the relapsed tumour.

Seven (35%) tumours before hormone relapse and 13 (65%) after ($P=0.11$) had increased copies of the AR (Table 1). In one case the primary tumour had increased copies of AR, but only one copy in the relapsed tumour.

Amplification of the AR gene was detected in one (5%) primary and three (15%) hormone-relapsed tumours (Table 1); there was therefore no significant difference in the level of AR gene amplification when primary and hormone-relapsed tumours were compared ($P=0.6$). There was no association between the Gleason sum in the primary or relapsed tumour and AR amplification. From the three patients with amplified AR in the hormone-relapsed tumour, one primary tumour had a Gleason sum of 9, two a Gleason sum of ≤ 6 , and two relapsed tumours had a Gleason sum of ≥ 8 and one a Gleason sum of 7. A further two hormone-relapsed tumours had high AR:X ratios (1.39 and 1.41), but did not appear to be amplified (ratio < 1.5). From the patients with amplified hormone-relapsed tumours, two had undergone orchidectomy and three had received anti-androgen therapy. Therefore, the type of treatment received did not appear to influence AR amplification.

Serial PSA levels were used to document the hormonal response and relapse in all patients (Fig. 1). In patients where there were no AR abnormalities down-regulation of PSA by hormonal therapy and subsequent PSA escape occurred as expected (e.g. Fig. 1a). In the case illustrated the biopsy after relapse was obtained following a 270-fold rise in PSA over 14 months (Fig. 1a). In the one case with amplification of AR in biopsies both before and after relapse (Fig. 1b) the PSA level rose from 0.9 $\mu\text{g/L}$ to 1450 $\mu\text{g/L}$ during hormone escape, with no increase in amplification of AR. Finally, the PSA response in one of two cases with amplification of AR in the hormone-relapse biopsy only is shown in Fig. 1c.

Discussion

Hormone-refractory prostate cancer has been postulated to develop because of amplification of the AR gene [9]. Several studies supporting this hypothesis investigated levels of AR gene amplifications and aneusomy of chromosome X in recurrent tumours from patients who initially responded to androgen-deprivation therapy [4,9,11,12]. Visakorpi *et al.* [9] reported that 30% of recurrent tumour specimens resistant to androgen-deprivation therapy had AR gene amplification. Although this level of amplification was too low to be the sole cause of hormone-refractory prostate cancer, it was suggested that an increase in AR copy number from either AR amplification or duplication of the X chromosome might be responsible. Visakorpi *et al.* [9] reported that a further 35% of tumours investigated were

Table 1 Cases with abnormal X chromosome or AR gene copy number, or an abnormal AR:X chromosome copy number ratio

Patient no.	X chromosome, biopsy		AR, biopsy		AR:X ratio	
	before (mid)	after relapse	before (mid)	after relapse	before (mid)	after relapse
2	1.63	1.58	1.61	1.63		
3	1.17 (1.06)	1.41	1.05 (1.07)	1.33		
5	1.32	1.46	1.42	1.48		
6	1.03	1.68	1.05	2.33	1.02	1.39
7	2.99 (2.72)	1.5	8.68 (11.87)	4.63	2.90 (4.36)	3.09
8	1.03	1.61	1.03	1.61		
9	1.46	1.04	1.48	1.04		
10	1.13	1.58	1.1	1.48		
13	1.07	1.53	1.08	2.15	1.01	1.41
16	1.22	1.79	1.22	1.85		
17	1.48	1.43	1.54	1.53		
18	1.17	1.25	1.2	1.32		
19	1.38	1.1	1.46	2.65	1.06	2.45
20	1.34	1.35	1.31	6.38	1.00	4.90

The mean X chromosome copy number per nucleus is given for each case if either of the biopsies before or after relapse had a mean chromosomal copy number of ≥ 1.3 . The mean AR gene copy number per nucleus is given for each case if either of the biopsies before or after relapse had a mean gene copy number of ≥ 1.23 . The mean AR:X chromosome ratio is given for each case that had an AR:X chromosome ratio of ≥ 1.07 , as above this value is considered abnormal. Cases were considered as having AR amplification if their mean AR:X chromosome ratio was > 1.5 .

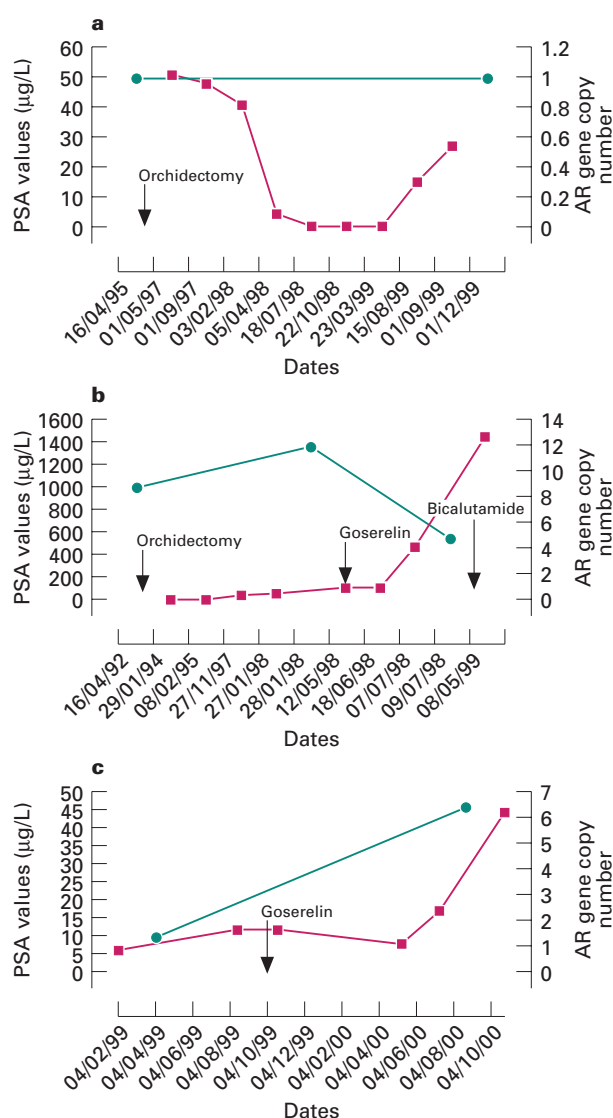


Fig. 1. a, The PSA profile for patient no. 1, showing elevated PSA levels at diagnosis, which reduced in response to treatment and increased when androgen-deprivation therapy failed. This patient, who initially responded to androgen-deprivation therapy, had no abnormalities in biopsies either before or after relapse for X chromosome or AR copy numbers. b, The PSA profile for patient no. 7, showing a sharp increase in PSA levels at relapse. There was no available value for PSA at diagnosis, but the records showed that PSA was elevated during this period. This patient responded to androgen-deprivation therapy and had AR amplification in biopsies both before and after relapse. c, The PSA profile for patient no. 20, showing high PSA levels at diagnosis, which increased until treatment was given and then decreased until relapse. This patient responded to androgen-deprivation therapy and had AR amplification only in the biopsy taken after relapse. In all figures the data-points shown as green circles are the mean AR copy number in each biopsy and those shown as red squares the PSA profile.

polysomic for chromosome X. Therefore, 65% of the recurrent cases investigated had increased AR copy numbers compared with normal prostate gland cells [9]. These findings have since been confirmed by several studies reporting amplification of the AR in 20–30% and polysomy of chromosome X in 35–80% of recurrent tumours [9,11,12]. These data have been used to suggest that an increase in AR copy number is involved in the development of hormone-refractory prostate cancer.

In the present study we have, for the first time, investigated AR amplification and copy number in paired tumours before and after hormonal escape in patients with documented initial responses to antiandrogen therapy. The results cast doubt on the role of AR amplification or duplication as mechanisms of hormone escape. Although 65% of recurrent tumours had an increase in AR copy number, consistent with values previously reported [9,11,12], 35% of them also had increased copies of AR in the pretreatment tumours. Despite this, each of these patients had a documented response to antiandrogens (Fig. 1), suggesting that the increased AR copy number did not predict androgen insensitivity. Furthermore, the mean AR copy number after relapse was no higher than that seen in the primary tumours (before therapy). Most strikingly, only three patients had amplification of the AR gene after hormone relapse; of these, one (5% of primary biopsies) had amplification of the AR gene before antiandrogen therapy. This patient, despite having the greatest amplification found in the study both in tumours before and after relapse, had a full PSA response to antiandrogen therapy. In this patient the PSA relapse could not be related to increased gene amplification of the AR (Fig. 1b).

These results, in which neither increased AR copy number nor amplification preclude a response to antiandrogen therapies, and where relapse is clearly not associated with either increased copy number or amplification of AR, call into question the role of alterations in AR copy number in hormone relapse in prostate cancer.

Although an increase in AR gene copy number does not seem to explain the development of hormone-refractory prostate cancer, we cannot, at present, exclude a change in the level of AR protein expression. Increases in AR expression may lead to hormone resistance and studies are currently underway to address this aspect of AR function in these samples. More recently it has been suggested that the development of hormone-resistant prostate cancer need not involve increased levels of AR but may involve mechanisms which alter AR function with no changes in expression. Recent evidence shows that activation of protein kinase A can up-regulate the expression of androgen-regulated genes via the

transcription factors cyclic AMP binding protein and activation transcription factor, by androgen-independent activation of AR [14,15] or via pathways that do not involve AR [16]. The protein kinase C pathway has also been reported to stimulate the expression of androgen-regulated genes via AP-1, an AR-independent signal transduction pathway [16,17]. Activation of either of these pathways would stimulate prostate tumour growth in the absence of circulating androgens, but whether any of these pathways are involved in androgen escape remains to be determined.

A lack of understanding of the mechanisms driving antiandrogen escape in prostate cancer remains central to the failure to develop viable alternative therapies for patients with hormone-relapsed disease. By investigating paired tumour samples before and after hormone relapse we provide evidence that questions the role of AR amplification in the process of hormone escape. As discussed above, alternative pathways may need to be investigated and could provide novel therapeutic targets for this important disease.

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Abbreviation: AR, androgen receptor.