Background: Androgen receptor (AR)-gene amplification, found in 20–30% of castration-resistant prostate cancer (CRPCa) is proposed to develop as a consequence of hormone deprivation therapy and be a prime cause of treatment failure. Here we investigate AR-gene amplification in cancers before hormone deprivation therapy.

Methods: A tissue microarray (TMA) series of 596 hormone-naive prostate cancers (HNPCas) was screened for chromosome X and AR-gene locus-specific copy number alterations using four-colour fluorescence in situ hybridisation.

Results: Both high level gain in chromosome X (≥4 fold; n = 4, 0.7%) and locus-specific amplification of the AR-gene (n = 6, 1%) were detected at low frequencies in HNPCa TMAs. Fluorescence in situ hybridisation mapping whole sections taken from the original HNPCa specimen blocks demonstrated that AR-gene amplifications exist in small foci of cells (<600 nm, <1% of tumour volume). Patients with AR gene-locus-specific copy number gains had poorer prostate cancer-specific survival.

Conclusion: Small clonal foci of cancer containing high level gain of the androgen receptor (AR)-gene develop before hormone deprivation therapy. Their small size makes detection by TMA inefficient and suggests a higher prevalence than that reported herein. It is hypothesised that a large proportion of AR-amplified CRPCa could pre-date hormone deprivation therapy and that these patients would potentially benefit from early total androgen ablation.
such biomarkers, the TransAtlantic Prostate Group established a cohort of PCA patients whose disease was conservatively managed and where samples for biomarker analysis were taken before hormone withdrawal therapy. In fluorescence in situ hybridisation (FISH)-based analyses, this clinical cohort has already yielded the discovery that two copies of a TMPRSS2/ERG translocation are associated with extremely poor PCA-specific survival (Attard et al., 2008; Clark et al., 2008), and that PTEN gene loss in the absence of ERG or ETV1 gene rearrangements identifies a group of poor prognosis patients (Reid et al., 2010).

Proliferation and survival of PCA cells are critically dependent on androgen stimulation, with treatment being primarily based on conventional androgen deprivation (also referred to as castration). Such treatments can often result in dramatic remission. However, the disease invariably relapses forming castration-resistant PCA (CRPCa), which features hypersensitivity of androgen pathway signalling that may result from amplification or activating mutations of the AR gene. AR-gene mutation has been reported in around 20% (Taplin et al., 1995, 2003) and AR amplification in 20–30% of CRPCa (Koivisto et al., 1997; Linja et al., 2001; Haapala et al., 2007), and has been suggested to develop during hormone deprivation therapy (Visakorpi et al., 1995; Palmberg et al., 1997; Haapala et al., 2007; Waltering et al., 2012). In contrast, AR-gene amplification has been reported at a low frequency in primary hormone naive cancers (1%, 3 out of 293 tumours from five studies; Koivisto et al., 1997; Palmberg et al., 1997; Bubendorf et al., 1999; Brown et al., 2002; Edwards et al., 2003). However, these published studies were limited by their use of small patient numbers, minimal sampling strategies (e.g., a single 0.6 mm core extracted from an entire cancer (Bubendorf et al., 1999), no patient survival data, high Gleason samples (Koivisto et al., 1997; Edwards et al., 2003) and a basic definition of the AR-gene alteration using single colour AR FISH probes. A recent study by Taylor et al. (2010) using gene copy number data from Agilent CGH arrays found no AR amplification in 181 primary tumours. Knowledge of the timing of occurrence of AR-gene amplification is critical. If such alterations are indeed induced by androgen withdrawal, they may have limited relevance to first-line patient therapy. However, if they are present before therapy, they could help inform on patient treatment.

Here we have used four-colour FISH probes to characterise the diversity of AR-gene alterations in human prostate cancer (PCA), and to assess their occurrence and clinical significance in a series of 596 hormone naive cancers represented on a TMA by up to six cores per patient and linked to 10 years follow-up survival data. We have in addition interrogated a number of whole-block tissue sections to investigate the prevalence of AR copy number altered PCA within individual selected samples.

**RESULTS**

**AR-gene status in hormone naive prostate cancer (HNPCa).** To assess AR-gene copy number gain and amplification, we used a multi-colour probe system consisting of three differentially labelled probes at the AR-locus (see Figure 1) plus an X-Centromere probe to determine ChrX copy number. The observed AR status of each patient was assigned to one of five categories (Cat) depending on the ChrX copy number (categories 1–4) or the presence of AR-gene locus-specific copy number gain (Cat5; see Figure 1).

AR FISH status was determined for a TMA series of 596 patients that had not been treated with androgen withdrawal therapy before sample collection (Cuzick et al., 2006) Cat1 (n = 444), Cat2 (n = 127), Cat3 (n = 15) and Cat4 (n = four). AR-locus-specific (Cat3) amplification was observed in six patients; three of which had gain of more than five copies of AR, one had three copies and two exhibited duplications. Correlations with clinical variables demonstrated an association between AR category and increased Gleason score, increased baseline PSA and the percentage of cancer in the original diagnostic biopsy (P < 0.001 for each of these factors), and also with more advanced clinical stage (P = 0.025; Table 1). There was no significant difference in age between the different categories (P = 0.550).

Locus-specific amplification and high copy number AR gain correlates with poorer cancer-specific survival. Correlations with
outcome were first considered for patients in categories 1 to 4. Univariate analysis (Table 2) demonstrated consistently poorer PCa-specific survival in patients with an increased ChrX copy number when compared with patients with a single copy of ChrX. However, this association was not maintained in multivariate analyses that included Gleason score, PSA level, patient age and

| Cat1. | 1X | 1:1:1:1 |
|-------|----|---------|
| Cat2. | 2X | 2:2:2:2 |
| Cat3. | 3X | 3:3:3:3 |
| Cat4. | 6X | 6:6:6:6 |
| Cat5. | AR gene-locus specific amplification | 1:2:2:1 |

Figure 1. (A) Multi-coloured fluorescence in situ hybridisation (FISH) probes used for investigating androgen receptor (AR) gene locus copy number (relative probe positions shown and actual distances indicated): (i) blue probe spanning the AR-gene (blue arrow) consisted of bacterial artificial chromosomes (BACs) RP11-479J1, RP11-963N10, CTD-2155B10 labelled with Aqua PlatinumBright kit (Kreatech Diagnostics). (ii) Red probe telomeric to AR, BACs RP11-466E18 and RP11-768G22 visualised with Cy3. (iii) Green probe centromeric to AR, BACs RP11-414C19 and RP11-745E2 visualised with FITC. Chromosome X (ChrX) centromere probe (CenX) was a 500-bp PCR product as Warburton et al (1991), sonicated and directly labelled with PlatinumBright 647 kit (far-red; Kreatech Diagnostics). (B) Examples of the five categories (Cat1–5) of AR copy number alterations. Cat1–4 consisted of increasing numbers of ChrX: Cat5 consisted of locus-specific amplification of AR. We scored the tumours by counting the Green, Blue, Red and CenX signals. For example, Cat1 contain a single ChrX in normal male cells seen as single overlapping Green (G), Blue (B) and Red (R) spots, which numerically correlated with a single centromere X signal (X) (not shown). This normal male pattern was counted as 1:1:1:1 for the numbers of GBRX probes counted in a cell. Gain of the ChrX was inferred when multiples of this pattern were seen (Cat2–4). In contrast, Cat5 amplification of the AR-gene locus could be visualised as strings of coloured beads corresponding to the co-amplification of different combinations of the Green, Blue and Red signals. Amplifications could involve all three AR locus colours or be just multiple adjacent copies of the Green and Blue signals or Blue and Red signals (as shown here). Amplification of the Blue probe alone was not seen in these studies.
cancer Ki67 status (Table 2). General aneuploidy has been linked to poorer PCa survival (Taylor et al., 2010), it is not known whether the chromosome X gains were specific to X or a general genomic gain.

Locus-specific amplification (Cat5) was significantly associated with poorer PCa-specific survival (hazard ratio (HR) = 5.08 (95% CI = 1.85–13.95)) in univariate analysis when compared with patients with a single copy of the AR-gene (Figure 2). The association became more marked in multivariate analyses that took into account Gleason sum, PSA, age and Ki67 (HR = 3.71, 95% CI = 1.35–10.18). Overall, the results demonstrated a consistent correlation between AR copy number and poorer cancer-specific survival; however, because of the small number of Cat5 patients in these analyses (n = 6), the results involving analyses of these particular samples were considered to be anecdotal only.

### Table 1. AR FISH categories and clinical parameters

| Variable | 1 | 2 | 3 | 4 | 5 | \(\chi^2\) (P-value) |
|----------|---|---|---|---|---|------------------|
| Gleason score | 24 | 5 | 0 | 0 | 1 | 82.97 (<0.001) |
| PSA | 165 | 30 | 1 | 0 | 2 | 65.71 (<0.001) |
| Clinical stage | 133 | 23 | 2 | 0 | 2 | 17.59 (0.025) |
| Cancer in biopsy (%) | 129 | 17 | 0 | 0 | 3 | 17.83 (<0.001) |

**Table 2.** AR FISH category, PCa-specific and overall mortality

| AR FISH categories (n = 596) | Univariate PCa specific | Univariate overall | Multivariate PCa specific | Multivariate overall |
|-----------------------------|-------------------------|--------------------|---------------------------|---------------------|
| Cat1 | 1.00 | 1.00 | 1.00 | 1.00 |
| Cat2 | 1.84 (1.26–2.66) | 1.22 (0.96–1.56) | 1.14 (0.77–1.68) | 0.94 (0.72–1.22) |
| Cat3 | 3.97 (2.06–7.65) | 1.54 (0.86–2.75) | 1.14 (0.56–2.33) | 0.77 (0.42–1.42) |
| Cat4 | 7.92 (2.50–25.13) | 3.57 (1.33–9.59) | 1.06 (0.32–3.51) | 1.11 (0.40–3.08) |
| Cat5 | 5.08 (1.85–13.95) | 1.61 (0.60–4.32) | 10.73 (3.64–31.66) | 3.71 (1.35–10.18) |

Abbreviations: AR = androgen receptor; FISH = fluorescence in situ hybridisation; PSA = prostate-specific antigen. AR FISH category and Gleason score, PSA, clinical stage and % cancer in diagnostic biopsy.

### Focal origin of locus-specific AR-gene amplification.

For each of the Cat5 samples, only a single core of the 1–4 cores assessed by FISH exhibited locus-specific AR-gene amplification (Table 3) demonstrating heterogeneity of AR status in cancer from single prostates. In addition, one of the cores assigned to Cat5 (Table 3; sample 4, core 2) contained two adjacent areas of high copy number gain; one with Cat5 (GBRX/2552) and one with Cat4 containing four to six copies of the X chromosome (Figure 3). This indicated that distinct mechanisms of high-copy number AR gain can occur in the same cancer.

To investigate these observations further, sections were cut from Cat5 whole-block TURP samples where significant amounts of tissue remained (samples 3, 4 and 5, Table 3). AR FISH maps for samples 3 and 4 are shown in Figure 4A and B. For sample 4, we detected small regions (foci) of cells with Cat5 and Cat4 alterations: both of these foci had been selected in the TMA cores (Table 3). The Cat5 alterations represented about 1% in the cancer areas in this TURP specimen and had the same GB RX FISH pattern as found in the TMA core. For samples 3 and 5, we failed to detect areas of Cat5 cancer, although two small areas of Cat4 cancer were observed in sample 3.

In the TMA analysis, we also detected three additional cases that exhibited between one and four cells containing a locus-specific AR-gene amplification (see example in Supplementary Figure 1). These cases were not assigned Cat5 status because they did not meet the scoring threshold of containing at least 50 altered cells.
For example, sample 7 in Table 3 had three to five copies of ChrX on TMA analysis and had been scored as Cat4. However, in core four, a single cell was observed that had a locus-specific AR-gene amplification. The AR FISH map for this TURP specimen is shown in Figure 4C. Over 99% of the cancer contained either two copies or three copies of ChrX. In addition, four small areas contained higher copy number gains. One focus harboured five copies of ChrX, whereas three other foci contained Cat5 AR-locus-specific amplifications of >seven-fold. None of these areas of Cat5 amplification were near the sites selected for TMA cores. Survival time for this patient was 4.2 years. Cat5 foci were not detected in the other two whole-block TURP sections, which were still alive at last follow-up (12 and 14 years).

The presence of Cat4 and Cat5 alterations appear to be linked. Only four Cat4 samples were found in TMA analyses (0.7%), however, when combining TMA and whole TURP analysis, three of the seven samples containing Cat5 alterations (3, 4 and 7) also had areas of Cat4 cancer (see Table 2 and Figure 4). In two of these samples, foci of Cat4 and Cat5 foci were in immediately adjacent tumour areas (Figures 3 and 4C).

From these observations, we draw several conclusions. First, foci of cells harbouring locus-specific AR-gene amplification occur in fields of tumour containing ChrX copy number gain. Second, high copy number gains of ChrX (Cat4) and locus-specific gains in AR frequently occur together in separate regions of the same cancer. Third, in the cases that we examined, foci of cells containing Cat4 and Cat5 alterations represent only a small percentage (<1%) of the overall cancer cell population.

**DISCUSSION**

The genetically heterogeneous nature of cancer is well documented. However, different models exist to explain its origin and...
significance. One view is that cancer clones can be arranged in a hierarchical structure that is maintained by rare (\(B_{1i}n_{106}\)) 'tumour-propagating cells', also called 'cancer stem cells'. An alternative view is that a significant proportion (or even the majority) of individual cells that have undergone malignant transformation can propagate a cancer (Quintana et al., 2008; Shackleton et al., 2009). In the latter model, even small volume clones arising within a tumour may be significant if they possess a growth advantage. In breast cancer, inter-tumoural heterogeneity of HER2 amplification linked to HER2 overexpression has been documented, and evidence supports the idea that tumour progression may in some cases result from selection of HER2 overexpressing subclones present in the primary cancer (Cottu et al., 2008; Shackleton et al., 2009). In the latter model, even small volume clones arising within a tumour may be significant if they possess a growth advantage. In breast cancer, inter-tumoural heterogeneity of HER2 amplification linked to HER2 overexpression has been documented, and evidence supports the idea that tumour progression may in some cases result from selection of HER2 overexpressing subclones present in the primary cancer (Cottu et al., 2008; Shackleton et al., 2009). We now hypothesise that a similar model of progression arises in PCA: namely that subclones of cells harbouring AR gain/amplification and consequent overexpression arise in primary hormone naïve tumours. AR amplification could, in principle, be due to conditions of localised androgen insufficiency within a PCA as a result of, for example, poor tumour vascularisation, or a general requirement for increased androgen signalling in tumour cells, which could be growth limiting to the tumour. We propose that these clonal growths would have a survival advantage on initiation of hormone deprivation therapy, and could seed the formation of CRPC, with concurrent further amplification of the AR gene by similar mechanisms (Figure 5, route 1). This has implications for both continuous and intermittent androgen deprivation therapy (Salonen et al., 2013). In support of this concept, the current study has demonstrated the occurrence of AR-gene amplification in HNPCa and revealed a consistent association between AR copy number and poor cancer-specific survival. Previously, it has been shown that increases in AR transcript level (a change that has been associated with AR amplification (Koivisto et al., 1997; Linja et al., 2001; Edwards et al., 2003)) are the only consistent alteration associated with the development of resistance to castration, and it has been demonstrated that increasing levels of AR can confer resistance to castration by amplifying signal output from low levels of residual ligand (Waltering et al., 2009). In principle, clones of cells containing AR amplification could exhibit a growth advantage and gradually become the predominant cancer

Figure 4. AR FISH analysis of whole TURP-block sections of samples 3, 4 and 7 (see Table 3). Coloured areas indicate the FISH score in areas of tumour identified by pathologist examination of an adjacent H&E-stained slice. Non-coloured areas are not tumour and were not scored. White areas are Cat1 with a single ChrX. Yellow and green circles indicate positions of the 600 µm diameter cores taken from areas of tumour and normal, respectively, for TMA construction. Blue and red arrows highlight the small foci of Cat4 and Cat5 tumour, respectively. The nuclei pictures in the key area are from sample 7. (A) Sample 4, cores taken for TMA construction have by chance sampled both the Cat4 and Cat5 tumour areas; (B) sample 3, this sample had a Cat5 tumour on TMA analysis, however, no Cat5 tumour was visible on whole-block analysis but foci of Cat4 tumour were present; (C) sample 7, this sample had only a single AR-amplified cell on TMA analysis but on whole-block analysis both Cat4 and Cat5 tumour foci were found. In all cases, Cat5 tumour foci were less than 1% of the whole-block tumour area.
clone, even in the absence of treatment by castration (Figure 5, route 2).

A critical question is, what proportion of castration-resistant patients that harbour an AR amplification could be attributable to pre-existing tumour clones in primary untreated tumours? Published data report that around 20% of primary tumours including those diagnosed by TURP (Edwards et al, 2003; Reid et al., 2010) progress to CRPCa (Byar, 1972; Taplin et al., 1995; Edwards et al., 2003; Cuzick et al., 2006), and 20–30% of these CRPCa harbour an AR amplification (Koivisto et al, 1997; Palmberg et al, 1997; Bubendorf et al., 1999; Brown et al., 2002; Edwards et al., 2003). Therefore, 4–6% of primary tumours are predicted to progress to CRPCa that contain a detectable AR amplification. Our studies have found an AR amplification in 6 of 596 patients (1%), which is a substantially lower rate of detection. However, this discrepancy could be explained by the small size of the AR-amplified clonal growths, which were less than 1% of tumour volume on whole section analysis, combined with the limited sampling that is an inherent problem of TMAs.

When taken together with the poor survival of AR-amplified patients, these data suggest that a significant proportion of AR-amplified CRPCa could develop from small clonal growths of AR-amplified cells present in pre-treatment neoplasms. Patients harbouring such clones would be predicted to be inherently more resistant to conventional androgen ablation therapy and would be excellent targets for first-line therapy with drugs that cause AR deprivation therapy or (2) natural androgen insufficiency within the tumour environment.

When pathogenesis to prevention of castration resistant prostate cancer. *Prostate* 70: 100–112.

Brown RSD, Edwards J, Dogan A, Payne H, Harland SJ, Bartlett JMS, Masters JRW (2002) Amplification of the androgen receptor gene in bone metastases from hormone-refractory prostate cancer. *J Pathol* 198: 237–244.

Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G, Kallioniemi OP (1999) Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence in situ hybridization on tissue microarrays. *Cancer Res* 59: 803–806.

Byar DP (1972) Survival of patients with incidentally found microscopic cancer of the prostate: results of a clinical trial of conservative treatment. *J Urol* 108: 908–913.
Cox D, Oakes D (1984) Analysis of Survival Data. Chapman & Hall: London 201.

Cuzick J, Attard G, Jhavar S, Flohr P, Berney D, Koivisto P, Visakorpi T, Kallioniemi OP (2010) Integrative genomic profiling of human prostate cancer. Cancer Cell 18: 11–22.

De Bono JS, Ashworth A (2010) Translating cancer research into targeted therapeutics. Nature 467: 543–549.

Edwards J, Krishna NS, Grigor KM, Bartlett JMS (2003) Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer 89: 552–556.

Haapala K, Kuukasjarvi T, Hyytinen E, Rantala I, Helin HJ, Koivisto P, tornado PA (2007) Androgen receptor amplification is associated with increased cell proliferation in prostate cancer. Hum Pathol 38: 474–478.

Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Koordiz A, Visakorpi T, Kallioniemi OP (1997) Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 57: 314–319.

Linja MJ, Savinainen KJ, Saramäki OR, Tammela TL, Vessella RL, Visakorpi T (2001) Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 61: 3550–3555.

Melchior S, Hadaschik B, Thüroff S, Thüroff J (2009) Outcome of radical prostatectomy for incidental carcinoma of the prostate. BJU Int 103: 1478–1481.

Palmberg C, Koivisto P, Hyytinen E, Isola J, Visakorpi T, Kallioniemi OP, Tammela T (1997) Androgen receptor gene amplification in a recurrent prostate cancer after monotherapy with the nonsteroidal potent antiandrogen Casodex (bicalutamide) with a subsequent favorable response to maximal androgen blockade. Eur Urol 31: 216–219.

Qingtao E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, Morrison SJ (2008) Efficient tumour formation by single human melanoma cells. Nature 456: 593–598.

Reid AHM, Attard G, Ambrosiine L, Fisher G, Kovacs G, Brewer D, Clark J, Flohr P, Edwards S, Berney DM, Foster CS, Fletcher A, Gerald WL, Moller H, Reuter VE, Scardino PT, Cuzick J, De Bono JS, Cooper CS, Translational Prostate Group (2010) Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer. Br J Cancer 102: 678–684.

Salonen AJ, Taari K, Alas-Opaa M, Viitanen J, Lundstedt S, Tammela TLJ, the FinnProstate Group (2013) Advanced Prostate Cancer Treated with Intermittent or Continuous Androgen Deprivation in the Randomised FinnProstate Study VII: Quality of Life and Adverse Effects. Eur Urol 63: 111–120.

Scher HI, Beer TM, Higano CS, Anand A, Taplin M-E, Elsaithiou E, Rathkopf D, Shelly J, Yu EY, Alumkal J, Hung D, Hirmand M, Seely L, Morris MJ, Danila DC, Humm J, Larson S, Fleisher M, Sawyers CL, Prostate Cancer Foundation/Department of Defense Prostate Cancer Clinical Trials Consortium (2010) Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1–2 study. Lancet 375: 1437–1446.

Shackleton M, Quintana E, Fearon ER, Morrison SJ (2009) Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 138: 822–829.

Taplin M-E, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Bult SP (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332: 1393–1398.

Taplin M-E, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, Stadler W, Hayes DF, Kantoff PW, Vogelzang NJ, Small EL, Cancer and Leukemia Group B Study 9663 (2003) Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol 21: 2673–2678.

Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS et al. (2010) Integrative genomic profiling of human prostate cancer. Cancer Cell 18: 11–22.

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP (1995) In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 9: 401–406.

Waltering KK, Helenius MA, Sahu B, Manni V, Linja MJ, Jänne OA, Visakorpi T (2009) Increased expression of androgen receptor sensitizes prostate cancer cells to low levels of androgens. Cancer Res 69: 8141–8149.

Waltering KK, Urbanucci A, Visakorpi T (2012) Androgen receptor (AR) aberrations in castration-resistant prostate cancer. Mol Cell Endoerinol 360: 38–43.

Warburton PE, Greig GM, Haaf T, Willard HF (1991) PCR amplification of chromosome-specific alpha satellite DNA: definition of centromeric STS markers and polymorphic analysis. Genomics 11: 324–333.

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