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Novel, gross chromosomal alterations involving *PTEN* cooperate with allelic loss in prostate cancer

Alison HM Reid[1,2,*], Gerhardt Attard[1,2,*], Daniel Brewer[1], Susana Miranda[1], Ruth Riisnaes[1], Jeremy Clark[1], Lucy Hylands[1], Sue Merson[1], Roy Vergis[2], Charles Jameson[2], Søren Høyer[3], Karina Dalsgaard Sørenson[3], Michael Borre[3], Chris Jones[1], Johann S de Bono[1,2] and Colin S Cooper[1]

[1]*The Institute of Cancer Research, Sutton, Surrey, UK;* [2]*The Royal Marsden NHS Foundation Trust, Sutton, Surrey, UK and* [3]*Aarhus University Hospital, Skejby, Denmark*

There is increasing evidence that multiple chromosomal rearrangements occur in prostate cancer. PTEN loss is considered to be a key event in prostate carcinogenesis but the mechanisms of loss remain to be fully elucidated. We hypothesised that gross rearrangements may exist that cause disruption of the *PTEN* gene in the absence of genomic deletion. We therefore designed a novel fluorescence *in situ* hybridisation (FISH) assay with probes overlying regions 3′ and 5′ of *PTEN* and a third probe overlying the gene. We aimed to identify both genomic deletions and gross rearrangements of *PTEN* that would be overlooked by previously reported single-probe FISH assays. We proceeded to evaluate a tissue microarray with radical prostatectomy and trans-urethral resection of the prostate specimens from 187 patients. We identified *PTEN* genomic loss in 45/150 (30%) radical prostatectomy patients and 16/37 (43%) trans-urethral resection of the prostate patients. Importantly, our assay detected novel chromosomal alterations in the *PTEN* gene (characterised by splitting of FISH signals) in 13 tumours (6.9% of all prostate cancers; 21% of *PTEN*-lost cancers). All *PTEN*-rearranged tumours had genomic loss at the other allele and had no expression of PTEN by immunohistochemistry. *PTEN*-rearranged tumours were significantly more likely to have an underlying *ERG* rearrangement. Our assay differentiated loss of the probe overlying PTEN in isolation or in combination with either one of or both the probes overlying the 3′ and 5′ regions. This gave an indication of the size of genomic loss and we observed considerable inter-tumoural heterogeneity in the extent of genomic loss in *PTEN*-lost tumours. In summary, gross rearrangements of the *PTEN* locus occur in prostate cancer and can be detected by a 'break-apart' FISH assay. This observation could explain the absence of PTEN protein expression in a subgroup of tumours previously classified as having heterozygous genomic loss using single-probe traditional FISH assays.
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Recent reports of paired-end, massively parallel sequencing of the prostate cancer genome identified novel loss-of-function chromosomal rearrangements[1] in addition to the previously described oncogenic chromosomal rearrangements involving ETS and BRAF family genes.[2–4] For example, gross rearrangements of MAGI2 have been described and evaluated using a fluorescence *in situ* hybridisation (FISH) break-apart assay.[1] In this recent paper, *PTEN* has been described to be altered by chromosomal rearrangement but the alterations described in clinical samples resulted in complete loss of one *PTEN* probe FISH signal[1] (Figure 1a). Gross chromosomal alterations of *PTEN* without copy number loss have however been reported in breast cancer xenografts with underlying DNA repair defects.[5] Loss of PTEN function is common and considered to be an important event in prostate carcinogenesis.[6,7] Multiple

Correspondence: Professor JS de Bono, The Institute of Cancer Research and the Royal Marsden NHS Foundation Trust, 15 Cotswold Road, Sutton, Surrey SM2 5PT, UK.
E-mail: johann.de-bono@icr.ac.uk
*These two authors are joint first authors.
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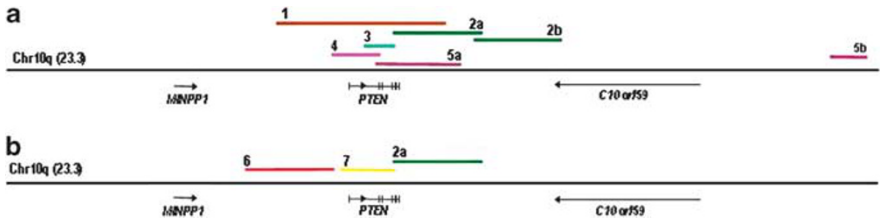

Figure 1 (**a**) FISH probes used in previous publications to detect *PTEN* gene loss. Probe 1 is a commercially available *PTEN*-specific probe (Vysis, Downers Grove, IL) previously used.[8,29,31] Probe 2a is BAC RP11-765C10 and 2b is BAC RP11-959L24 that map to the minimum region of *PTEN* deletion[22] and were used in combination in Reid *et al*.[9] Probe 3 is PAC190P6, covering *PTEN* exons 3–9 inclusive and mapping to the minimum region of deletion of *PTEN* and used in Verhagen *et al*.[10] Probe 4 is BAC CTD-2047N14 used to detect *PTEN* loss in Berger *et al*.[1] Probe 5a is BAC RP11-846G17 mapping to *PTEN* and probe 5b is BAC RP11-399O19 mapping to the flanking *FAS* gene. Both probes were used in Sircar *et al*.[21] The direction of gene transcription is indicated with arrows. *C10orf59* and *MINPP1* are two genes close to *PTEN*. (**b**) Novel *PTEN* FISH assay to detect rearrangements. At the 3′ end, the BAC probe (RP11-210E13, red) immediately flanks *PTEN* and at the 5′ end, BAC probe (RP11-765C10, green) partially covers *PTEN*. A further BAC probe over *PTEN* (CTD-2267G16, yellow) was used to investigate whether *PTEN* could be lost or rearranged in isolation of flanking regions.

mechanisms underlie loss of PTEN function[7] with genomic loss occurring in up to 40–50% of primary prostate cancers.[8–11] FISH studies allow the robust evaluation of genomic loss in reasonably sized tumour cohorts and importantly, most have identified an association between genomic *PTEN* loss and worse clinical outcome.[8,9] However, as most reported studies have utilised single FISH probes over *PTEN* (Figure 1a), alleles disrupted by gross chromosomal alterations without copy number loss may not have been detected and would have been wrongly classified as *PTEN* wild type. We therefore sought to develop a FISH assay that would comprehensively identify both copy number loss and gross chromosomal alterations of the *PTEN* locus; these were then evaluated in a cohort of prostate cancers identified in a PSA-screened population.

Materials and methods

Tissue Microarrays and Patient Cohort

Tissue microarrays were constructed, as previously described, from tissue obtained at the time of surgery.[12] Briefly, one or two 0.6-mm cores were taken from the donor block with a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Sections of 4 μm were cut on a microtome and transferred to glass slides (Menzel–Gläser, Superfrost, Braunschweig). Prostate cancer samples were collected between 6th June 1995 and 4th November 2005 by intended radical prostatectomy from men with histologically confirmed, clinically localised prostate cancer and by trans-urethral resection of the prostate from men with non-localised prostate cancer treated at the Department of Urology, Aarhus University Hospital, Skejby, Denmark. One hundred and ninety-five tumour cores from 150 patients treated by radical prostatectomy for localized prostate cancer and 37 patients treated by trans-urethral resection of the prostate for non-localized prostate cancer were used. Clinicopathological data on all patients was available and is presented in the results per the REporting recommendations for tumour MARKer prognostic studies (REMARK).[13] Additionally, high-grade glioma samples were collected from the archives of Kings College Hospital, London. In total, 389 cores from 342 patients were collated in four tissue microarrays. The cases comprised 276 glioblastoma multiforme (WHO grade IV), 17 anaplastic astrocytoma (WHO grade III), and 49 anaplastic oligodendroglioma (WHO grade III). Ethical approval for the collection of the cohorts was obtained from the Ethics Review Committees of the collaborating hospitals. Areas of 'cancer' and 'normal' were identified on the basis of histopathological examination of haematoxylin and eosin sections that flanked the tissue microarray slice used for FISH and immunohistochemistry studies and for the prostate cancer tissue microarray, p63/AMACR-stained sections were also available. BACs for probes were chosen using the UCSC genome browser (http://genome.ucsc.edu) and were labelled as described previously.[14,15] FISH studies for *PTEN* followed by rehybridisation using an *ERG* break-apart assay were then conducted as described previously.[9,15–17] Tissue microarrays were fluorescently scanned at $\times 20$ magnification on an Ariol SL-50 (Applied Imaging, San Jose, CA, USA) with a 5×0.5 μm *z*-stack, and images were stored and double scored by two operators (AR and GA/SM). FISH signals in a minimum of 200 nuclei were scored in each core, although often FISH signals in >1000 nuclei per core were assessed.

Immunohistochemistry

In all, 4 μm sections were cut and immunostained using a commercially available PTEN antibody (Cell Signaling Technology #9559) and standard heat-induced antigen retrieval methods were used. As all samples were collected at the same institution, variability of tissue fixation was minimised. Samples were, however, collected over a 10-year period,

Table 1 Distribution of patients by *PTEN* gene status using our traditional single-probe assay

Procedure (N)	*PTEN normal*	*PTEN loss*	*Heterozygous loss*		*Homozygous loss*	
	Total number (126)	*Total number (61)*	*Heterogeneous*	*Homogeneous*	*Heterogeneous*	*Homogeneous*
Radical prostatectomy (150)	105	45	6	0	19	20
TURP (37)	21	16	2	2	8	4

Abbreviation: TURP, trans-urethral resection of the prostate.

which may impact immunohistochemistry results. PTEN wild-type controls included normal prostate tissue and 22RV-1 xenograft, and PTEN-loss controls included PC3 (prostate cancer cell line—PTEN null) xenografts. For negative control slides, the primary antibody step was omitted and ChromPure rabbit IgG applied instead. Cytoplasmic PTEN staining was scored according to the product of staining intensity on a 0–3 scale multiplied by the percentage of immunoreactive cells in the cancerous areas. We considered any score of ≥ 0 to represent some degree of positive staining. Cases were analysed only if positive internal controls were present (ie cells that would be expected to stain positive).

Statistical Analysis

To classify a tissue microarray cancer core as having homozygous *PTEN* loss, simultaneous lack of both signals of *PTEN* gene locus-targeted probe and the presence of two signals of chromosome 10 centromeric probe had to occur in $\geq 10\%$ of nuclei. To classify a core as having heterozygous loss, $\geq 40\%$ of nuclei had to contain one signal of *PTEN* gene locus-targeted probe and two signals of chromosome 10 centromeric probes. These cutoffs were established in previous work where FISH signals were counted in normal and cancer nuclei.[9] To test significance, the appropriate statistical tool as specified in the text was applied (statistician: DB) and significance was defined as a two-sided *P*-value of <0.05. Time to biochemical failure was defined as a rise in PSA to >0.2 ng/dl was calculated for patients treated by radical prostatectomy using the Kaplan–Meier method.

Results

Novel Gross Alterations Disrupt the *PTEN* Locus in Prostate Cancer

We designed and optimised a FISH assay, hereinafter referred to as the 'break-apart' assay, to detect alterations at the *PTEN* gene locus. Two BAC probes were selected that mapped to the *PTEN* locus. At the 5′ end, a probe (RP11-765C10 labelled with FITC) partially covered *PTEN* and at the 3′ end, a probe (RP11-210E13 labelled with CY-3) immediately flanked *PTEN* (Figure 1b). A commercially available, aqua-labelled DNA chromosome 10 centromere probe (chromosome 10, p11.1∼q11.1 Abbott Molecular, Des Plaines) was used to ensure detection of *PTEN* loss when ploidy was present. Probes were tested on metaphase spreads to ensure that they did not cross-hybridise with any region other than the *PTEN* locus. Using the 'break-apart assay', *PTEN* loci without ploidy and without loss or rearrangement are visualised in interphase nuclei as adjacent red and green signals. Tissue microarrays from patients having had a radical prostatectomy or trans-urethral resection of the prostate (Table 1) were evaluated with both our single-probe 'traditional' *PTEN* FISH assay (as described previously (Figure 1a))[9] and our novel, comprehensive, 'break-apart' assay (Figure 1b). Two cancer-containing cores were available from eight patients and one core from the other 179 patients.

As reported previously, we devised cutoffs based on counting of *PTEN* probes in normal and cancerous prostatic tissue (see Statistical Analysis).[9] Using a single probe over *PTEN*, 105/150 patients treated by radical prostatectomy and 21/37 trans-urethral resection of the prostate cancers had no areas meeting the criteria for *PTEN* loss and were called 'normal'. Of the remaining 45 patients treated by radical prostatectomy, 20 had uniform (homogeneous) homozygous loss across the whole core. In keeping with previous reports of tumour heterogeneity in prostate cancer,[17–19] the other 25 tumours had a heterogeneous pattern with areas of both homozygous and heterozygous *PTEN* loss: 19 heterogeneous tumours were classified as homozygous loss tumours and six as heterozygous (cutoffs defined in Statistical Analysis) (Table 1). Of the remaining 16 patients treated by trans-urethral resection of the prostate, four had homogeneous homozygous loss and two had uniform heterozygous loss. Of the remaining patients, 10 had a heterogeneous pattern: eight classified as homozygous loss and two as heterozygous loss.

Using the 'break-apart assay', chromosomal alterations resulting in breakpoints along the *PTEN* gene and characterised by splitting of FISH signals in the absence of copy number loss were observed in 13 tumours that all had genomic loss at the other allele (6.9% of all prostate cancers and 21% of *PTEN*-lost cancers) (Figure 2). In this series, we did not detect any cases with splitting of signals in the absence of

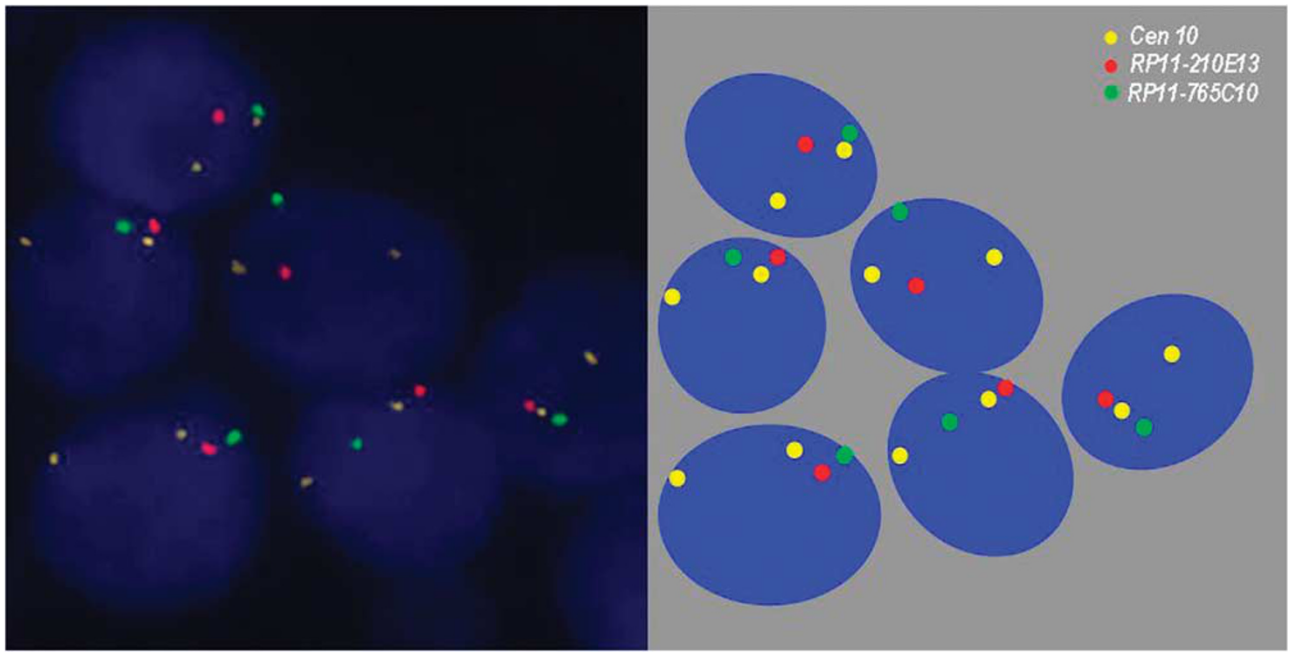

Figure 2 Gross alterations of the *PTEN* gene. In the picture on the left-hand side, DAPI-stained nuclei are seen with the BAC probe (RP11-210E13) immediately flanking *PTEN* (red) and the BAC probe (RP11-765C10) partially covering *PTEN* (green) (described in Figure 1b). A centromere 10 probe in yellow was also used. Two copies of the centromere probe are seen in each nucleus with only one copy of each of the *PTEN* probes 'split' from each other. The probes from the other *PTEN* allele are completely lost. A cartoon representation of the nuclei with gross *PTEN* alterations is seen on the right-hand side.

Table 2 PTEN immunohistochemistry scores for patients distributed by underlying *PTEN* FISH status

	PTEN *normal* (N = 76)[a]	PTEN *loss by biallelic genomic deletion* (N = 37)[b]		PTEN *loss by genomic deletion and chromosomal rearrangement* (N = 12)[c]
		Heterogeneous loss	*Homogeneous loss*	
IHC result				
Positive, total (%)	32 (42)	4 (22)	1 (5)	0
Negative, total (%)	44 (58)	14 (78)	18 (95)	12 (100)

[a]Data for 50 patients not available for analysis.
[b]Data for 14 patients missing.
[c]Data for 1 patient missing.

genomic loss. Using this assay, the operators scored a median of 236 (range: 215–296) nuclei to obtain informative FISH results from 200 nuclei.

Chromosomal Alterations Involving *PTEN* Cooperate with Allelic Loss in Prostate Cancer

To confirm that splitting of signals using our 'break-apart' FISH assay resulted in loss of PTEN protein, we proceeded to evaluate PTEN status by immunohistochemistry in all tumours acquired by either radical prostatectomy or trans-urethral resection of the prostate. In patients with neither genomic loss nor gross chromosomal alterations of *PTEN*, 42% of patients were classified as having positive staining of PTEN by immunohistochemistry and 58% were completely negative (Table 2). In some tumours, PTEN status varied across the core—for example, areas with underlying genomic *PTEN* loss (heterozygous or homozygous as detected by FISH) have been observed adjacent to each other and adjacent to areas of normal PTEN. Therefore, tumours with areas of retained PTEN loci could have positive immunohistochemistry staining in addition to negative staining and we described this as 'heterogenous'. As homozygous loss was heterogenous in 27 tumours (Table 1) and those cells in a core with retained PTEN loci could result in positive immunohistochemistry staining, we differentiated tumours with heterogenous from those with homogenous loss (Table 2). In fact, 22% of tumours with heterogeneous homozygous genomic loss stained positive by immunohistochemistry and 78% stained

negative. In contrast, 5% of tumours with homogeneous homozygous loss stained positive and 95% were negative by immunohistochemistry. All tumours with heterozygous genomic loss and an associated chromosomal alteration were negative by immunohistochemistry. There was no statistically significant association with outcome when tumours with PTEN loss detected by immunohistochemistry were compared with tumours without PTEN loss (data not shown). Interestingly, a core was found that had an area of cancer with heterozygous genomic loss and a chromosomal rearrangement adjacent to an area with normal *PTEN* FISH; the latter area was positive on immunohistochemistry and the former area was negative (Figure 3).

Table 3 reports patient demographics and clinicopathological variables for radical prostatectomy tumours with wild-type *PTEN* FISH, tumours classified as homozygous genomic loss (both heterogeneous and homogeneous) and tumours with heterozygous genomic loss associated with a chromosomal alteration (homozygous loss secondary to a chromosomal alteration).

There was a significant association between homozygous loss of *PTEN* by either biallelic genomic deletion or genomic deletion in combination with chromosomal alteration and clinical tumour stage ($P=0.023$ and 0.005, respectively; χ^2 test for trend) but the association with Gleason score was not significant ($P=0.36$; χ^2 test) (Table 3). Complete data for clinical nodal stage was not available. There was no association with median PSA or age ($P>0.1$; Wilcox *t*-test). Of the 150 patients treated with radical prostatectomy, 57 had a biochemical recurrence. Eight patients received adjuvant hormones and were not included in the following analysis. For the remaining 141 patients, 5/6 (83%) of tumours with homozygous loss secondary to genomic loss and a rearrangement relapsed; 20/34 (59%) of tumours with biallelic *PTEN* loss relapsed; and 47/99 (47%) of cases with normal *PTEN* status had relapsed. The predicted median time to biochemical recurrence was 1114 days (95% CI = 695 to unestimatable) for *PTEN* tumours with homozygous loss secondary to genomic loss and a rearrangement; 1192 days (95% CI = 786 to unestimatable) in biallelic *PTEN* loss; and 1464 days (95% CI = 1089 to unestimatable) in *PTEN* normal cases. There was no statistically significant difference between the three groups in this series ($P>0.05$).

Footprint of Genomic *PTEN* Loss Is Variable

We then rehybridised a series of cores using a probe directly over the *PTEN* gene (CTD-2267G16, yellow), in addition to the two 'break-apart' assay probes (RP11-210E13 and RP11-765C10), to establish whether *PTEN* could be lost or rearranged in isolation of flanking regions (Figure 1b). The probe over *PTEN* was lost either with the 3′-centromeric probe (red) or with the 5′-telomeric probe (green) but was never lost in isolation. In three cases, the probe directly over *PTEN* appeared to split, half associating with the red and half with the green probe (Table 4A). In addition to identifying novel rearrangements of *PTEN* using the 'break-apart' FISH assay, we also observed that the extent of *PTEN* loss varies between patients. In 80% of cases, the pattern of *PTEN* loss was the same for all alleles (there were cases with ploidy) in each cell. Three patterns of *PTEN* genomic loss were observed: (1) complete loss of all three probes over *PTEN* (region of deletion $\geq$483 kb); (2)

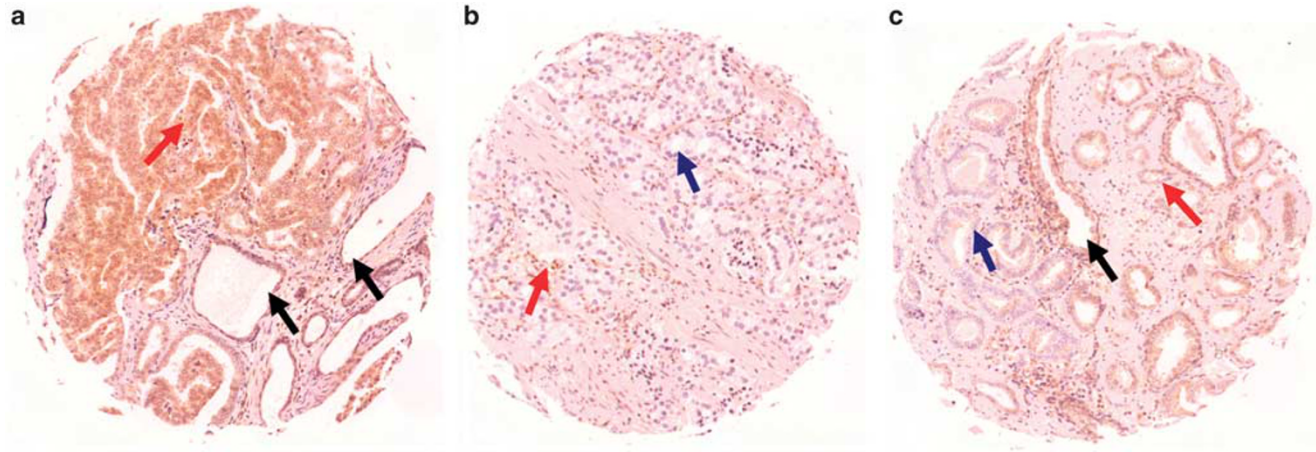

Figure 3 Tissue microarray cores immunostained with an antibody against PTEN. (**a**) A cancer core with evidence of strong positive PTEN staining in the cancer glands (indicated with red arrow). The normal glands (indicated with a black arrow) also stain positively but less intensely than the cancer glands. (**b**) A cancer core with loss of PTEN staining in cancer glands (indicated with a blue arrow) and control endothelial cells staining positively (indicated with a red arrow). (**c**) One-half of the core has cancer glands, which stain negatively for PTEN (indicated with a blue arrow). FISH with the 'break-apart' assay revealed heterozygous genomic loss plus a chromosomal rearrangement in this area. The other half of the cancer glands have a normal PTEN complement (indicated with a red arrow) and FISH revealed two wild-type PTEN copies in this area. Finally, the gland in the middle of the core is a normal gland, which also stains positively (indicated with a black arrow) and retained both PTEN alleles, as expected, by FISH.

Table 3 Patient demographics and clinicopathological features

	PTEN normal (N = *105*)	*PTEN homozygous loss by biallelic genomic loss* (N = *38*)		*PTEN homozygous loss by genomic loss and chromosomal rearrangements* (N = *7*)	
Median PSA (range)	15.3 (1.9–211)	11.25 (2.1–54.1)	*P*-value >0.1[a]	15 (7–47)	*P*-value >0.1[a]
Median age (range)	63 (48–78)	62 (52–76)[b]	*P*-value >0.1[a]	63 (49–68)	*P*-value >0.1[a]
Clinical T stage					
Total T1 (%)	45 (43)	8 (21)[c]	*P*-value = 0.023[d]	1 (14)	*P*-value = 0.005[d]
Total T2 (%)	58 (55)	26 (68)		2 (29)	
Total T3 (%)	2 (2)	2 (5)		4 (57)	
Total T4 (%)	0	0		0	
Gleason grade					
Total <7 (%)	49 (47)	5 (13)	*P*-value = 0.007[e]	3 (43)	*P*-value = 0.36[e]
Total ≥7 (%)	56 (53)	32 (84)[b]		4 (57)	

[a]Wilcox *t*-test.
[b]Data for 1 patient missing.
[c]Data for 2 patients missing.
[d]χ^2 test for trend.
[e]χ^2 test.
[f]Fisher's exact test.
P-values refer to significance of difference compared with PTEN normal.

Table 4A PTEN FISH break-apart patterns with three-probe assay (Figure 1)

FISH pattern	*Number of cases*
Probes 6+7 split from probe 2a	5
Probe 6 split from probes 7+2a	5
Probe 6+part of probe 7 split from part of probe 7+probe 2a	3

Table 4B Footprint of PTEN loss detected using three-probe assay in cases with PTEN loss but no rearrangement

FISH pattern	*Proportion (%) of PTEN lost cases*
Complete loss of all three *PTEN* probes (region of deletion ≥483 kb)	62.5%
Loss of probe 2a+7 but maintenance of probe 6 (region of deletion ~283 kb)	27.5%
Loss of only probe 2a with maintenance of probes 6+7 (region of deletion ~183 kb)	10%

loss of the probe 5′ to *PTEN* (green) and the probe directly over *PTEN* (yellow) but maintenance of the 3′ probe (red) (region of deletion ~283 kb); (3) loss of only the 5′ probe (green) with maintenance of the 3′ probe (red) and the probe (yellow) directly over *PTEN* (region of deletion ~183 kb) (Table 4B). In the remaining 20% of cases, a heterogeneous different pattern of *PTEN* loss was observed. For example, we observed complete loss of all three probes in one allele and loss of only the 5′ probe with maintenance of the 3′ probe and the probe directly over *PTEN* in the other allele in three tumours and similarly, complete loss of all three probes in one allele and loss of the 5′ *PTEN* probe and the probe directly over *PTEN* but maintenance of the 3′ probe in the other allele in two tumours.

PTEN Genomic Aberrations Are Associated with Rearrangements of *ERG*

The distribution of tumours with an *ERG* rearrangement with respect to *PTEN* status is shown in Table 5. We observed a significant association between *PTEN* loss and *ERG* gene rearrangements both secondary to biallelic genomic loss ($P = 0.0004$; χ^2 test) and genomic loss in association with a chromosomal rearrangement ($P = 0.0187$; χ^2 test).

PTEN Loss Occurs Commonly in Glioblastoma Multiforme but Gross Chromosomal Alterations Are Rare

We report *PTEN* rearrangements in prostate cancer and they have also been reported in breast cancer xenografts[5] albeit not to date in breast cancer clinical samples. We therefore proceeded to evaluate whether rearrangements occur in glioblastoma, another cancer type with a well-documented high incidence of PTEN loss.[20] Heterozygous loss of *PTEN* was observed in 119/253 (47%) assessable glioblastoma cases, with homozygous deletions seen in a further 10 cases (3.9%). There were no correlations between *PTEN* loss and clinical outcome. A single case with a split *PTEN* FISH signal was observed in a 67-year-old female patient with glioblastoma. Heterozygous loss of *PTEN* was seen in 2/13 anaplastic astrocytoma (15.4%) and 6/41 anaplastic

Table 5 *ERG* gene rearrangement distribution across three classes of *PTEN* loss

ERG rearrangement status	*PTEN normal (*N = *107)*[a]	*PTEN homozygous loss (*N = *46)*[b]	*PTEN homozygous loss secondary to chromosomal rearrangements (*N = *13)*
Patients with an ERG *rearrangement*			
Total (%)	55 (51)	38 (83)	11 (85)
Class Edel (%)	30 (28)	22 (48)	7 (54)
Class Esplit (%)	25 (23)	16 (35)	4 (31)
Patients without an *ERG* rearrangement (%)	52 (48)	8 (17)	2 (15)

[a]Data for 19 patients missing.
[b]Data for 5 patients missing.
Class Edel = the rearrangement is associated with deletion of sequences 5′ to *ERG* with retention of a single 3′-*ERG* signal.
Class Esplit = the rearrangement is characterised by retention but separation of probe signals from the (3′) and (5′) probes.

oligodendroglioma (14.6%); there were no homozygous deletions. Therefore, only one case in a series of 307 high-grade glioma samples contained a split *PTEN* pattern.

Discussion

We present a novel 'break-apart' *PTEN* FISH assay that detects both gross chromosomal rearrangements and genomic deletion. Previous studies of *PTEN* with FISH have mostly used single probes overlying the gene, which have not detected rearrangements. Sircar *et al*[21] have used a combination of BAC probes to detect *PTEN* and two flanking genes (*BMPR1A* and *FAS*) (part shown in Figure 1b) but the probe overlying *PTEN* was again single and rearrangements were not detected with this assay, suggesting that the rearrangements we report in this study occur in a relatively small area around the *PTEN* locus. In our series, there were no deletions of *PTEN* involving solely our middle probe (CTD-2267G16) (Figure 1b). This is in keeping with previous studies that reported a minimal region of *PTEN* loss[22] covered by our 5′ probe (RP11-765C10). If this observation is confirmed by ongoing analyses in larger data sets, it could be sufficient to proceed with the three-colour 'break-apart' assay using the 3′ (RP11-210E13) and 5′ (RP11-765C10) probes over *PTEN* together with a reference probe. Our study confirms the presence of loss-of-function gross chromosomal rearrangements in prostate cancer that are similar to recently described rearrangements that involve MAGI2.[1] All the chromosomal rearrangements in our series occurred in tumours with genomic loss at the other allele(s). This observation also requires evaluation in a larger series of tumours to investigate whether gross chromosomal rearrangements could occur in the absence of copy number loss. We showed that tumours with heterozygous genomic deletion plus a chromosomal rearrangement had complete loss of expression of PTEN protein. Immunohistochemistry is subject to operator and processing bias and there is no universally established assay for assessing PTEN protein expression. We have developed an immunohistochemistry protocol (described in Materials and methods) that has given robust results. Importantly, there was significant loss of PTEN protein in tumours with homogeneous homozygous *PTEN* genomic loss, with a 'false-positive' rate of 5%. This could be explained by tumour heterogeneity and in fact, tumours that had heterogeneous homozygous loss by FISH (we used the adjacent tissue slice or <5 slices apart for immunohistochemistry and FISH) were more likely to show areas of PTEN immunohistochemistry positivity (Table 3). Immunohistochemistry results can also be influenced by technical challenges such as variable tissue fixation and slide storage before staining. We also here report complete loss of PTEN protein expression in 58% of tumours that were *PTEN* wild type by FISH, in keeping with previous reports of multiple alternative mechanisms for loss of PTEN protein.[7] Assessments with clinical outcome in this series were limited by the duration of follow-up and size of this cohort. Although some reports have described a worse outcome for tumours with homozygous compared with heterozygous genomic *PTEN* loss,[8] we have previously reported no difference between these two groups.[9] This could now be explained by tumours with homozygous loss secondary to a chromosomal rearrangement being wrongly classified as having heterozygous *PTEN* loss using a single-probe assay.

We also report here significant heterogeneity of *PTEN* loss in primary prostate cancers and to account for this and also sectioning artefacts, we compared FISH probe patterns in nuclei in normal tissue to those in cancers with a range of patterns. We then defined tumours with $\geq 10\%$ of nuclei showing loss of all *PTEN* probes as homozygous and tumours with $\geq 40\%$ of nuclei showing loss of one probe but preservation of the other allele as heterozygous (loss of both probes is less likely to be due to chance than loss of solely one probe, hence the lower proportion of nuclei required to exclude an artefact). The probe over the chromosome 10 centromere also assists in assessing whether a *PTEN* allele is truly lost as opposed to sectioning or hybridisa-

tion artefact. This strict classification may classify cores with a small area of *PTEN* loss as *PTEN* normal. Tumours with both homozygous and heterozygous loss were classified as homozygous, recognising the hypothesis that tumour clones with homozygous loss are more aggressive and will outgrow other areas.

PTEN is increasingly undergoing evaluation as a biomarker for identifying poor prognosis patients and selecting patients for targeted therapies, including the plethora of new agents targeting the PI3K AKT pathway[23] and DNA repair proteins.[24,25] Moreover, the identification of gross chromosomal rearrangements could also give important insights to tumour biology with reports increasingly suggesting that a subset of advanced solid cancer types are characterised by a propensity to acquire chromosomal rearrangements during their development.[1] This is not common to all solid tumours as suggested by our observation that *PTEN* loss in GBM is very rarely associated with a gross chromosomal rearrangement. In preclinical models, loss of *PTEN* can cooperate with *ERG* rearrangements to promote prostate cancer progression[26,27] and in fact several studies have now reported an association between *ERG* rearrangements and *PTEN* loss identified by FISH.[28,29] We also report here a similar significant association between *PTEN* loss with a gross chromosomal rearrangement and an *ERG* gene rearrangement. The previous observation of *PTEN* chromosomal rearrangements in breast cancer xenografts with an underlying DNA repair defect[5] and emerging evidence implicating DNA repair defects in the development of chromosomal rearrangements in prostate cancer[1] suggests that demonstration of chromosomal rearrangements in a subset of tumours could be exploited for patient selection for therapeutic agents targeting DNA repair defects.[30] Therefore, although chromosomal rearrangements disrupting *PTEN* are uncommon and may not occur in the absence of genomic deletion at the other allele, complex assays similar to ours that accurately map the *PTEN* locus should be considered for future *PTEN* FISH studies.

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Disclosure/conflict of interest

Several of the authors are employed by The Institute of Cancer Research that has a commercial interest in the development of PI3K inhibitors.

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