Integrated analysis of the genomic instability of *PTEN* in clinically insignificant and significant prostate cancer

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Patients with clinically insignificant prostate cancer remain a major over-treated population. *PTEN* loss is one of the most recurrent alterations in prostate cancer associated with an aggressive phenotype, however, the occurrence of *PTEN* loss in insignificant prostate cancer has not been reported and its role in the separation of insignificant from significant prostate cancer is unclear. An integrated analysis of *PTEN* loss was, therefore, performed for structural variations, point mutations and protein expression in clinically insignificant (48 cases) and significant (76 cases) prostate cancers treated by radical prostatectomy. Whole-genome mate pair sequencing was performed on tumor cells isolated by laser capture microdissection to characterize *PTEN* structural alterations. Fluorescence *in situ* hybridization probes were constructed from the sequencing data to detect the spectrum of these *PTEN* alterations. *PTEN* loss by mate pair sequencing and fluorescence *in situ* hybridization occurred in 2% of insignificant, 13% of large volume Gleason score 6, and 46% of Gleason score 7 and higher cancers. In Gleason score 7 cancers with *PTEN* loss, *PTEN* alterations were detected in both Gleason pattern 3 and 4 in 57% of cases by mate pair sequencing, 75% by *in situ* hybridization and 86% by immunohistochemistry. *PTEN* loss by sequencing was strongly associated with *TMPRSS2-ERG* fusion, biochemical recurrence, *PTEN* loss by *in situ* hybridization and protein loss by immunohistochemistry. The complex nature of *PTEN* rearrangements was unveiled by sequencing, detailing the heterogeneous events leading to homozygous loss of *PTEN*. *PTEN* point mutation was present in 5% of clinically significant tumors and not in insignificant cancer or high-grade prostatic intraepithelial neoplasia. *PTEN* loss is infrequent in clinically insignificant prostate cancer, and is associated with higher grade tumors. Detection of *PTEN* loss in Gleason score 6 cancer in a needle biopsy specimen indicates a higher likelihood of clinically significant prostate cancer.

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The tumor suppressor gene, *PTEN* (phosphatase and tensin homolog on chromosome 10), a phosphoinositide 3-phosphatase, negatively regulates the PI3K/AKT signaling pathway and functions as a tumor suppressor. It is one of the most commonly altered genes in prostate cancer. Mutations in *PTEN* occur in

up to 10% of primary prostate cancers while *PTEN* deletion occurs in 10–70% of surgically treated cancers and over 50% of metastatic prostate cancers.^{1–11} *PTEN* deletion is associated with poorer cancer specific outcomes, and there is an association of deletion with increasing stage and Gleason score.^{7,9,12–16} Although associated with later stage and higher grade tumors, the frequency of *PTEN* loss is reported in 3–10% of Gleason score 6 cancers, and ~15% of confined pT2 tumors.^{12,15} In regards to the proposed precursor lesion, high-grade prostatic intraepithelial neoplasia, the data are inconsistent and limited with five studies to date. In three studies

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that used fluorescence *in situ* hybridization for the evaluation of *PTEN* status, deletion was identified in 9, 13, and 23% cases of high-grade prostatic intraepithelial neoplasia.^{7,10,17} These findings in addition to mouse models that showed the development of high-grade prostatic intraepithelial neoplasia-like lesions following *PTEN* inactivation through deletion and mutation suggested that *PTEN* loss is an early event in prostate carcinogenesis, and potentially associated with more aggressive disease.^{18–21} However, a study by McMenamin *et al* and by Lotan *et al* using immunohistochemistry indicate that in high-grade prostatic intraepithelial neoplasia, *PTEN* protein is intact suggesting that *PTEN* loss is a later event.^{22,23}

As *PTEN* loss is associated with aggressive prostate cancer, it could potentially have a role in separating patients that have significant prostate cancer from those with insignificant cancer. However, there remain important questions in regards to *PTEN* deletion and mutation and risk of prostate cancer significance and progression. Although *PTEN* deletion is reported in Gleason score 6 and confined prostate cancer, the frequency of *PTEN* mutation and deletion is unknown in insignificant prostate cancer (defined in radical prostatectomy specimens as tumors that are Gleason score 6, confined, and tumor volume < 0.5 cm³). In addition, recent studies of contemporary Gleason grading and studies of Gleason score 6 cancer and outcome following surgery, suggest that studies have potentially overestimated the frequency of *PTEN* deletion in Gleason score 6 cancer by under-grading Gleason score 7 and higher grade cancers.²⁴ Recently the presence of *PTEN* protein loss in Gleason score 6 needle biopsy specimens has been associated with increased likelihood of Gleason score upgrading in radical prostatectomy specimens.²⁵ Understanding the relationship of *PTEN* loss, cancer significance and the relationship of Gleason pattern 3 and Gleason pattern 4 in regards to shared *PTEN* alterations in Gleason score 7 and higher cancer has important implications in the assessment of prostate needle biopsy specimens where only a small portion of a tumor is sampled.

The primary objectives of this study were to characterize the *PTEN* status in insignificant and significant prostate cancer from radical prostatectomy specimens using whole-genome mate pair sequencing, Sanger sequencing, fluorescence *in situ* hybridization and immunohistochemistry to determine the frequency of *PTEN* abnormalities in insignificant and significant prostate cancer including structural alterations and mutations. The additional objective was to understand the relationship of Gleason pattern 3 and Gleason pattern 4 in Gleason score 7 cancers in regard to *PTEN* loss to determine if understanding *PTEN* status could have a role in determining prostate cancer significance and stratifying risk of progression in newly diagnosed prostate cancer patients.

Materials and methods

Tissue Collection and Processing

Consecutive cases of insignificant Gleason score 6 (48 cases), large volume Gleason score 6 (23 cases), Gleason score 7 (29 cases), and Gleason score 8 and higher cancers (25 cases) were selected from the Mayo Clinic prostate cancer frozen repository. Insignificant prostate cancer was defined as Gleason score 6, stage pT2 and tumor volume < 0.5 cm³. Processing of radical prostatectomy specimens occurred as previously described.²⁶ All specimens were handled in the fresh state and analyzed initially with a frozen microtome technique. The prostate was inked, serially sectioned in the horizontal plane, and right apex, left apex and bladder base margins (perpendicular) were examined microscopically followed by standard sections from the inferior, mid and superior posterior prostate and right and left vesicles. In cases of insignificant Gleason score 6 cancer, the entire posterior prostate was examined with additional sections of the right and left anterior prostate. The freezing microtome technique conserves tissues such that a top and bottom created from cutting allows for the collection of fresh insignificant prostate cancer (unfrozen top section collected for analysis). For large volume Gleason score 6, Gleason score 7 and Gleason score 8 and higher tumors, the dominant index tumor in the gland was collected. The tops and bottoms of the tissue cut by the microtome technique were processed to formalin-fixed paraffin-embedded tissue sections for review after routine processing.

Isolation of DNA and Mate Pair Sequencing

Tumor was collected by laser capture microdissection (Arcturus instrument) from 10 µm unstained fresh-frozen sections and DNA amplified directly from cells, as previously described.^{27–30} In Gleason score 7, the Gleason pattern 3 and Gleason pattern 4 were collected and analyzed separately.²⁸ Libraries were prepared using the Illumina Mate Pair (MP) Kit following the manufacturer's instructions and sequenced as two libraries per lane on the Illumina HiSeq platform.^{27–31} Data were processed using previously described binary indexing mapping algorithms developed in our group.³² The read-to-reference-genome-mapping algorithm was modified to map both mate pair sequencing reads across the whole genome. Discordant mapping mate pair sequencing reads that were covered by at least five associates were identified for further analysis. Concordant mapping mate pair sequencing reads were used to determine frequency coverage levels across the genome.^{31,32}

Fluorescence *In Situ* Hybridization

The diverse repertoire of *PTEN* structural variants detected by whole-genome mate pair sequencing was

used to develop a unique *PTEN* fluorescence *in situ* hybridization probe set that would detect as many structural variants as possible in the corresponding formalin-fixed paraffin-embedded prostate cancer. Probes were designed and prepared in a similar manner to probes developed in our laboratory. Briefly, bacterial artificial chromosomes corresponding to the regions of interest overlying *PTEN* and adjacent regions were obtained from ResGen Invitrogen. DNA was isolated using the QIAGEN Plasmid Maxi Kit and labeled using the Vysis Nick Translation kit and either SpectrumGreen-dUTP or SpectrumOrange-dUTP (Vysis). Each BAC clone was tested individually by hybridization to normal metaphases. Polymerase chain reaction confirmed clones covered the regions of interest. Centromere 10 status was assessed using commercially available probe (CEP10, Abbott Molecular). Tissue sections (5 μ m) were deparaffinized at 90 °C, immersed twice in xylene, twice in 100% ethanol, dried, microwaved in 10 mmol/l citric acid (10 min), pretreated with 2x saline-sodium citrate buffer (5 min), digested (40 min) with DigestALL (Invitrogen) and washed. Slides were denatured at 80 °C (5 min) and hybridized at 37 °C overnight in humidified incubator. DAPI1 (10%; Abbott) was used for counter staining. Before hybridization, tumor was identified and labeled by a pathologist on H&E slides that corresponded with sections used for *in situ* hybridization. Each case was scored by two technicians in a blinded manner. Before scoring, technicians first scanned the entire region of interest to identify potential *PTEN* status heterogeneity within the tumor. Then the cases were scored with each technician scoring 100 interphase nuclei from representative areas. Each cell was scored for all probes simultaneously. Scoring was done blinded to whole-genome mate pair sequencing, Sanger sequencing, and immunohistochemical results. In Gleason score 7 cancer, Gleason pattern 3 and Gleason pattern 4 were scored separately.

Immunohistochemistry

Formalin-fixed paraffin-embedded 5 μ m sections from the same tissue block used for *in situ* hybridization were incubated in EDTA-containing antigen retrieval buffer (32 min), followed by application of primary rabbit monoclonal antibody (*PTEN* clone D4.3; Cell Signaling) at 1:200 dilution made with Dako Background Reducing Diluent for (32 min) at 37 °C. Immunostaining was performed on the Ventana BenchMark XT Stainer with Ventana Optiview Plus Amp detection system followed by Ventana DAB, hematoxylin (8 min) and bluing agent (4 min). Cancer was scored independently by two pathologists (JCC and EPC) as the percentage of cells showing markedly decreased or absent *PTEN* staining, and the tumor was considered to show *PTEN* loss by immunohistochemistry if any area (or percentage of) tumor lacked *PTEN* staining. Scoring

was done blinded to whole-genome mate pair sequencing, fluorescence *in situ* hybridization and Sanger sequencing results. In Gleason score 7, the Gleason pattern 3 and Gleason pattern 4 were scored separately.

Sanger Sequencing

Tumor and associated normal prostate DNA (10 ng) purified from the laser capture microdissected fresh-frozen tissue was used to assess *PTEN* gene mutation status. Bi-directional Sanger sequence analysis was performed to test for the presence of a mutation in all coding regions and intron/exon boundaries of the *PTEN* gene (GenBank accession number NM_000314; NG_007466.2).

Results

Clinical and Pathological Features

Clinico-pathologic features of the patients were available for 110 (87%) of the cases (Table 1). As expected preoperative serum PSA and pathological tumor stage correlated with Gleason score. The mean tumor size for the large volume Gleason score 6 was similar to that of the Gleason score 7 group at 3.9 and 3.7 cm³, respectively, whereas the Gleason score 8 and higher tumors had a significantly greater mean volume at 10.4 cm³. By definition, all insignificant Gleason score 6 cancers were < 0.5 cm³. Clinical outcome data demonstrated that biochemical recurrence and systemic progression correlated with Gleason score (Table 1). Three (3%) patients died of prostate cancer, all from the Gleason score 8 and higher group. Survival curves with biochemical recurrence as an end point exemplify the stratification of insignificant and large volume Gleason score 6 from higher Gleason score cancers (Figure 1).

Large Genomic Rearrangements of *PTEN*

Whole-genome mate pair sequencing provided in-depth profiling of large genomic rearrangements potentially affecting *PTEN* function in the 126 prostate cancers. In total, 29 (23%) cases showed genomic alterations that predicted loss of function of *PTEN*. A detailed description of genomic breakpoints affecting chromosome 10 and the *PTEN* locus is provided in Table 2 and depicted in Figure 2. Polymerase chain reaction validations on selected *PTEN* deletions confirmed the validity of detected events (Supplementary Figure 1). The majority of cases with *PTEN* abnormalities (92%) fell in the Gleason score 7 and 8 and higher groups (14 (48%) and 11 (44%) cases, respectively). Just a single insignificant Gleason score 6 cancer (2%) and three large volume Gleason score 6 (13%) cancers had *PTEN* loss. Overall, events were more common in higher Gleason grade tumors, detected in 11 (11%) of

Table 1 Clinico-pathological features

	Gleason score 6 insignificant (N = 38)	Gleason score 6 large volume (N = 23)	Gleason score 7 (N = 25)	Gleason score 8+ (N = 24)	P-value
<i>Age at surgery</i>					0.0006
Mean (s.d.)	57.7 (6.9)	60.7 (6.9)	63.2 (6.0)	65.8 (7.4)	
Median	58	60	62	66	
Range	(42.0–71.0)	(51.0–74.0)	(51.0–75.0)	(51.0–81.0)	
<i>Preoperative PSA (ng/ml)</i>					0.0086
Mean (s.d.)	5.5 (2.5)	6.8 (2.6)	12.0 (14.1)	10.3 (6.5)	
Median	4.9	6.4	6.7	9.1	
Range	(1.2–12.2)	(3.9–14.6)	(2.5–54.4)	(2.5–23.2)	
<i>Pathological T-stage</i>					0.0001
2A	16 (42.1%)	5 (21.7%)	3 (12.0%)	1 (4.5%)	
2B	20 (52.6%)	16 (69.6%)	11 (44.0%)	10 (45.5%)	
2C	2 (5.3%)	0 (0.0%)	2 (8.0%)	0 (0.0%)	
3A	0 (0.0%)	2 (8.7%)	5 (20.0%)	5 (22.7%)	
3B	0 (0.0%)	0 (0.0%)	4 (16.0%)	6 (27.3%)	
<i>Estimated tumor volume (cc)</i>					< 0.0001
N	36	20	24	22	
Mean (s.d.)	0.3 (0.2)	3.9 (4.1)	3.7 (4.2)	10.4 (10.8)	
Median	0.3	2.8	2.5	7.5	
Range	(0.0–0.7)	(0.8–18.3)	(0.3–19.7)	(1.2–37.7)	
Biochemical failure	1 (2.6%)	1 (4.3%)	12 (48.0%)	17 (70.8%)	Total 31 (28.2%)
Systemic progression	0 (0.0%)	0 (0.0%)	4 (16.0%)	7 (29.2%)	11 (10.0%)
Death due to PC or 30 day death	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (12.5%)	3 (2.7%)
Death of any cause	1 (2.6%)	0 (0.0%)	3 (12.0%)	3 (12.5%)	7 (6.4%)

Figure 1 Survival curves with biochemical recurrence as an end point exemplifying the stratification of insignificant Gleason 6 and large volume Gleason score 6 from higher Gleason score cancers. Percentage of patients free of biochemical recurrence (y-axis) plotted against years from surgery (x-axis) for four patient groups; patients with insignificant tumor (solid gray line), all Gleason score 6 tumors (solid black line), large volume Gleason score 6 together with Gleason score 7+ tumors (dashed gray line) and Gleason score 7 and higher tumors (dashed black line).

101 Gleason pattern 3 cancers and 25 (47%) of 53 Gleason patterns 4/5 cancers.

Mate pair reads spanning *PTEN* and predicting deletions were detected in 26 of the 29 cases (Table 2). *PTEN* deletion events ranged from single deletion events spanning the *PTEN* gene, to more complex rearrangements, with multiple rearrangements across the chromosome 10q23 region. In

many cases multiple breakpoint events occurred surrounding *PTEN*, predicting more complex rearrangement events leading to loss of *PTEN*, with these more complex events more commonly present in Gleason pattern 4 than Gleason pattern 3. In addition to intrachromosomal rearrangements, interchromosomal translocations occurred adjacent to *PTEN*, often in association with intrachromosomal events.

Table 2 Summary of mate pair data in impacted cases

	Case number	Gleason score	Gleason pattern	Event	PTEN expression prediction	Intrachromosomal					Interchromosomal					Chromosome partners (+Number of events)	Shared PTEN events	PTEN copies*	TMPRSS2-ERG fusion	Lineage	PTEN Sanger mutation
						Hit Chr10	Hit Chr10q	Span PTEN	Hit 10q23	Hit PTEN	Hit Chr10	Hit Chr10q	Hit 10q23	Hit PTEN							
Significant Lineage —related or no PTEN event in Gleason pattern 3	11	7	3	N	None	0	0	0	0	0	0	0	0	0	-	NA	2	Yes	High	No	
	11	7	4	CD	Loss	9	9	5	7	1	4	4	3	0	9		1	Yes	High	Yes	
	24	7	3	N	None	0	0	0	0	0	1	1	0	0	8	NA	1	Yes	High	No	
	24	7	4	D	Loss	3	3	1	2	1	1	1	0	0	8		0	Yes	High	No	
	28	7	3	N	None	0	0	0	0	0	0	0	0	0		NA	2	Yes	High	No	
	28	7	4	T	ND	0	0	0	0	0	1	1	1	0	14		0	Yes	High	No	
	30	7	3	D	Loss	0	0	0	0	0	2	1	0	0	2, 3	Yes	1	Yes	High	Yes	
	30	7	4	DD	Loss	2	2	2	1	1	3	3	0	0	2, 3 ²		1	Yes	High	Yes	
	33	7	3	N	None	5	5	0	1	0	3	2	0	0	1, 11, 12	Yes	2	Yes	High	No	
	33	7	4	C+T	Loss	2	2	2	2	1	7	6	2	1	1, 3, 11, 12, 13 ³		1	Yes	High	No	
	13	7	3	D	Loss	3	2	1	2	0	5	4	4	0	3	Yes	0	Yes	High	No	
	13	7	4	D	Loss	1	1	1	1	1	1	1	0	0	3		0	Yes	High	Yes	
	21	7	3	D	Loss	4	3	2	2	0	3	3	2	0	5 ² 14	Yes	1	Yes	High	No	
	21	7	4	D	Loss	6	5	1	1	0	6	3	1	0	4,5,6,8,11,17		1	Yes	High	No	
	26	7	3	D	Loss	1	1	1	1	0	0	0	0	0		Yes	0	Yes	High	No	
	26	7	4	D	Loss	1	1	1	1	0	1	1	0	0	2		0	Yes	High	No	
	119	7	3	T	Loss	5	4	2	1	0	9	9	5	1	3 ² ,6,7 ² ,12,17 ³	Yes	0	Yes	High	No	
	119	7	4	C+T	Loss	3	3	1	0	0	8	8	4	0	3 ³ , 7 ² , 17 ³		0	Yes	High	No	
	64	7	3	D	ND	1	1	1	1	0	1	0	0	0	5	Yes	2	No	Med	No	
	64	7	4	D	ND	6	3	1	1	0	5	0	0	0	5 ⁴ 4		1	No	Med	Yes	
No/Low lineage and no shared PTEN	23	7	3	D	Loss	1	1	1	1	1	0	0	0	0		No	1	Yes	Low	No	
	23	7	4	C+T	Loss	12	12	10	11	0	2	2	2	1	8, 9		0	Yes	Low	No	
	31	7	3	Inv	Loss	4	4	3	4	3	0	0	0	0	-	No	2	Yes	Low	No	
	31	7	4	C	Loss	4	4	3	3	2	2	2	0	0	8		2	Yes	Low	No	
	35	7	3	N	None	0	0	0	0	0	0	0	0	0	-	No	2	Yes	None	No	
	35	7	4	C	Loss	22	7	3	5	0	30	26	3	0	3 ²⁸ 1 ²		0	No	None	No	
	62	7	3	CD	Loss	6	5	1	1	0	7	6	2	0	1, 3 ² ,5,6,7,12	No	1	No	None	No	
	62	7	4	T	ND	1	1	0	0	0	1	1	1	0	2		1	No	None	Yes	
Single Gleason pattern samples	103	8+	4/5	D	Loss	3	3	1	1	0	4	3	0	0	1,4 ³	NA	1	Yes	NA	No	
	104	8+	4/5	T	Loss	1	1	0	1	0	5	5	5	0	4	NA	1	Yes	NA	No	
	105	8+	4/5	DD	Loss	8	8	4	5	0	6	6	4	0	1 ³ , 5 ² , 8	NA	0	No	NA	No	
	113	8+	4/5	T	Loss	1	0	0	0	0	5	3	1	0	5 ³ , 6 ²	NA	1	No	NA	No	
	115	8+	4/5	CD	Loss	28	27	13	11	3	4	4	0	0	7	NA	1	No	NA	No	
	116	8+	4/5	CD	Loss	5	5	1	2	1	1	1	0	0	12	NA	1	Yes	NA	No	
	118	8+	4/5	DD	Loss	3	3	1	2	1	1	1	0	0	1	NA	0	No	NA	No	
	120	8+	4/5	D	Loss	1	1	1	1	0	0	0	0	0		NA	1	No	NA	Yes	
	121	8+	4/5	CD	Loss	8	8	4	5	0	0	0	0	0	-	NA	0	Yes	NA	No	
	124	8+	4/5	CD	Loss	8	8	5	7	2	23	23	18	1	3 ²¹ 4 ²	NA	1	No	NA	No	
	128	8+	4	CD	Loss	27	24	1	7	0	6	4	0	0	1, 3 ² , 7, 8, 9	NA	1	Yes	NA	No	
	38	6LV	3	D	Loss	2	2	1	0	0	4	0	0	0	3 ² ,13,14	NA	1	Yes	NA	No	
	41	6LV	3	CD	Loss	3	2	2	2	0	0	0	0	0	-	NA	1	Yes	NA	No	
	49	6LV	3	D	Loss	2	2	1	1	0	4	3	2	0	13 ² , 16 ²	NA	1	Yes	NA	No	
	65	6INS	3	CD	Loss	5	5	2	5	1	9	9	7	0	5, 14 ⁶ , 20 ²	NA	1	No	NA	No	

Abbreviations: C, complex event; CD, complex deletion; DD, double deletion; INS, insignificant cancer; Inv, inversion; LV, large volume; N, no events; NA, not applicable; ND, not determined; T, translocations.

*Predicted number of copies factoring in tumor cellularity.

Figure 2 Rearrangements within the 10q23 locus. **(a)** Repertoire of rearrangements observed in *PTEN* in the prostate tumors studied from whole-genome mate pair sequencing. *PTEN* region is presented spanning from 84 to 98 Mb of 10q23 with the position of *PTEN* indicated. Rearrangements for specific tumors listed are presented as deletions (solid lines), inversions (dashed lines), or spans of collective complex events (arrowed triple lines). Specific intrachromosomal breakpoints across 10q23 are marked by circles and numbers indicate translocation to different chromosome. Arrowed lines indicate additional events outside the 10q23 region presented. Cases are presented with alternative gray and black coloring for clarity. In the upper Gleason score 7 group, a and b refer to the Gleason pattern 3 and Gleason pattern 4 components, respectively. **(b)** Positioning of *PTEN* probes designed for fluorescence *in situ* hybridization.

Multiple potentially independent events within a single tumor indicated homozygous loss of *PTEN*. Genomic breakpoints hitting directly within the *PTEN* gene-coding region were observed in 16 tumors, 13 involving rearrangements within chromosome 10 and 4 by interchromosomal translocations fusing the *PTEN* coding region to different

chromosomes. Although the effect of these alterations on *PTEN* function is sometimes difficult to interpret, copy number variation levels from the mate pair sequencing data confirmed the loss of the *PTEN*. Homozygous loss of *PTEN* was predicted in nine tumors, all Gleason score 7 and higher. Chromothripsis or chromosomal catastrophe is a single

catastrophic event resulting in multiple genomic rearrangements, and associated with poor outcome in a number of cancers. When defined by the following: affected locus >10 megabases, number of clustered breakpoints >12, breakpoints involving one or two chromosomes, and inclusion of alternating copy number states, insertions and loss of heterozygosity, none of the cases showed involvement of the 10q23 region as we have previously reported.²⁹

In Gleason score 7 tumor with *PTEN* loss (14 cases), all Gleason pattern 4 had rearrangements involving *PTEN*, compared with 8 (57%) of the corresponding Gleason pattern 3. In these eight cases, 5 (63%) shared identical *PTEN* events within Gleason pattern 3 and Gleason pattern 4, whereas three cases contained unique events in the Gleason pattern 3 and the Gleason pattern 4. Unique events in *PTEN* were often seen in addition to shared lineage events, indicative of tumor progression. This observation was also evident in coverage frequency plots across *PTEN*, with additional deletion events in Gleason pattern 4 in the background of events in the Gleason pattern 3, indicative of progressive bi-allelic loss of *PTEN* (Supplementary Figure 2).

In addition to evaluation of *PTEN* status in the tumor, whole-genome mate pair sequencing was performed on high-grade prostatic intraepithelial neoplasia collected by laser capture microdissection. High-grade prostatic intraepithelial neoplasia was collected from 30 patients consisting of 15 insignificant Gleason score 6, six large volume Gleason score 6, and nine Gleason score 7 and higher cases. High-grade prostatic intraepithelial neoplasia was collected in the same section as tumor but where it was clearly delineated from the cancer to allow collection by laser capture microdissection without contamination by tumor. Rearrangements resulting in *PTEN* loss were not observed in any cases of high-grade prostatic intraepithelial neoplasia.

Heterogeneity of *PTEN* Loss and Correlation with *TMPRSS2-ERG* Fusion

TMPRSS2-ERG fusion was present in ~50% of cases from the mate pair sequencing data (Table 2). Although not predictive of tumor grade, *TMPRSS2-ERG* fusion correlated with *PTEN* loss (Figure 3a). Specifically in Gleason score 6 and Gleason score 7 cancer, nearly 80% of cases with loss of *PTEN*, manifested a *TMPRSS2-ERG* fusion. However, correlation was lower in the Gleason score 8 and higher group where just a 45% correlation was observed, consistent with previous reports.¹⁵

Consistent with previously reported data from our group, identical *TMPRSS2-ERG* fusions were present in both Gleason pattern 3 and Gleason pattern 4 in 11 of the 14 Gleason score 7 cases²⁹ (Table 2). An overall assessment of lineage between the Gleason pattern 3 and Gleason pattern 4 in Gleason score 7

cancer utilizing all shared rearrangements enabled assignment of high, medium, low or no lineage calls (Table 2). Overall, unique *PTEN* events in Gleason pattern 3 and Gleason pattern 4 correlated with lineage. For example, in cases PR23, PR31, and PR62, where no shared *PTEN* events were observed between the Gleason patterns in a tumor, the lineage between these patterns was assessed low or none. Conversely, all high/medium lineage cases presented with either shared identical *PTEN* events or no events in Gleason pattern 3 and Gleason pattern 4.

PTEN Point Mutations

To investigate the involvement of point mutations in the *PTEN* gene, each exon and the promoter element of every tumor, high-grade prostatic intraepithelial neoplasia and associated normal prostate tissue of the 126 cases were screened by bi-directional Sanger sequencing. Six (5%) cases possessed mutations in the *PTEN* gene (Table 2 and Table 3), five Gleason score 7 tumors and one Gleason score 9 tumor. Just one of the mutations in the Gleason score 7 group was present in the Gleason pattern 3 component, and in the remaining four; the point mutation was in Gleason pattern 4 only. All of the cases with *PTEN* point mutation presented with additional *PTEN* loss by mate pair sequencing (Table 2) indicating a mechanism of homozygous loss of *PTEN* in 5% of cases. No mutations were observed in high-grade prostatic intraepithelial neoplasia or normal tissues.

Evaluation of *PTEN* Rearrangements by Fluorescence *In Situ* Hybridization

Guided by the array of structural alterations identified by whole-genome mate pair sequencing (Figure 2), a fluorescence *in situ* hybridization probe set was developed to validate the *PTEN* rearrangements and performed on formalin-fixed paraffin-embedded tissues from 97 of the 126 cases. The *PTEN in situ* hybridization results showed high concordance with the mate pair sequencing results (Figure 3b). Twenty-two of the 29 cases with *PTEN* deletion by mate pair sequencing were evaluated by fluorescence *in situ* hybridization with equivalent Gleason grades observed in the formalin-fixed paraffin-embedded tissues (Table 4). Of these 20 (91%) showed *PTEN* alterations by both *in situ* hybridization and mate pair sequencing. The two cases that did not correlate were both from Gleason score 7 group (Supplementary Table 1). In the remaining cases where no *PTEN* deletion was detected by mate pair sequencing, 10 (10%) showed *PTEN* loss by fluorescence *in situ* hybridization (Table 4) with the majority in the Gleason score 6 groups (Figure 3b). In addition, in Gleason score 7 tumors with *PTEN* loss, Gleason pattern 3 and Gleason pattern 4 shared loss in 75% of cases. This

Figure 3 Detection of *PTEN* loss by mate pair sequencing, fluorescence *in situ* hybridization (FISH) and immunohistochemistry. Percentage of cases in the insignificant Gleason score 6, large volume Gleason score 6, Gleason score 7, Gleason score 8 and higher or all tumor groups associated with: (a) *TPRSS2-ERG* Fusion (*ERG* positive) and *PTEN* loss by mate pair sequencing. Cases with *TPRSS2-ERG* fusions (light gray bars), *PTEN* loss (black bars) or both (dark gray bars). (b) *PTEN* loss by mate pair sequencing (black bars), FISH (light gray bars) or both (dark gray bars). (c) *PTEN* loss by mate pair sequencing (black bars), immunohistochemistry (light gray bars) or both (dark gray bars). (d) *PTEN* loss by mate pair sequencing (black bars), FISH (light gray bars), immunohistochemistry (dark gray bars), or shared loss by two (hatched bar) or three (dotted bar) techniques. (e and f) Comparison of *PTEN* loss by MP, FISH and immunohistochemistry in cases where all three techniques applied as measures by any loss (e) or as single and double copy losses (f).

Table 3 *PTEN* mutations by Sanger sequencing

Case	Gleason score	PTEN Sanger mutation			PTEN status Gleason pattern 3	PTEN status Gleason pattern 4
		Gleason patterns	Mutation	Protein		
PR13	7	4	1007 dup A	p.Y336YX	Deletion	Deletion+Mutation
PR30	7	4+3	497 dup T	p.T167NfsX13	Deletion+Mutation	Deletion+Mutation
PR62	7	4	987 990 del TAAA	p.N329KfsX14	Deletion	Mutation
PR64	7	4	334C>G	p.L112V	Intact	Deletion+Mutation
PR11	7	4	892C>CT	p.Q298QX	Intact	Deletion+Mutation
PR120	9	4+5	306 het del A	p.K102NfsX11	NA	Deletion+Mutation

Table 4 Concordance between mate pair, fluorescence *in situ* hybridization (FISH) and immunohistochemistry

	FISH			Immunohistochemistry		
	N	D	P-value	N	D	P-value
Mate pair						
N	64 (66%)	10 (10%)	< 0.0001	69 (64%)	12 (11%)	< 0.0001
D	3 (3%)	20 (21%)		3 (3%)	23 (21%)	
			N = 97			N = 107
Immunohistochemistry				Concordance:		
N	60 (63%)	7 (7%)	< 0.0001	Mate Pair: FISH		87%
D	7 (7%)	22 (23%)		Mate Pair: Immunohistochemistry		86%
			N = 96	Immunohistochemistry: FISH		85%

is the result of limited sampling of the laser capture microdissected samples compared with scanning the entire tumor in fluorescence *in situ* hybridization resulting in the identification of subpopulations within Gleason pattern 3 that show *PTEN* loss.

Immunohistochemical Staining for *PTEN*

Immunohistochemical staining for *PTEN* was additionally performed on the formalin-fixed paraffin-embedded tissues from 107 (85%) of the 126 cases studied by mate pair sequencing. The tumor was considered to show loss of *PTEN* if any tumor showed markedly decreased or absent staining. Loss of *PTEN* staining was observed in 35 (33%) cases (Figure 3c and Table 4) with the majority of tumors (95%) showing >10% loss. In regards to staining heterogeneity, six (17%) cases showed <10% staining loss, six (17%) showed 10–50% staining loss, and 23 (66%) showed >50% staining loss. Of the 29 cases with *PTEN* loss by mate pair sequencing, 26 were assessed by immunohistochemistry, with 23 (89%) confirming a loss of *PTEN* expression (Figure 3c). The three cases where no loss of *PTEN* expression was observed by mate pair sequencing consisted of one case each from the insignificant Gleason score 6, large volume Gleason score 6, and Gleason score 7 groups (Supplementary Table 1).

The insignificant Gleason score 6 case (PR65) also showed intact *PTEN* by fluorescence *in situ* hybridization. Of the remaining 82 cases surveyed, 12 (11%) showed loss of *PTEN* expression in the absence of detected mate pair sequencing rearrangements. From these 12 cases, 10 were also analyzed by fluorescence *in situ* hybridization, with five showing loss of *PTEN*. In addition, by immunohistochemistry, 86% of cases with *PTEN* loss in the Gleason pattern 4 also observed loss in the adjacent Gleason pattern 3. No *PTEN* expression loss was observed in high-grade prostatic intraepithelial neoplasia.

Concordance of *PTEN* Loss by Mate Pair Sequencing, Fluorescence *In Situ* Hybridization, and Immunohistochemistry

The concordance in detection of *PTEN* loss between each technique was high, ranging from 85.4 to 86.6% (Table 4). Concordance between the three techniques was higher in the higher grade (Gleason score 7 and higher) tumors, with less concordance in Gleason score 6 (Figure 3d) with additional cases of *PTEN* loss detected by both fluorescence *in situ* hybridization and immunohistochemistry that were not detected by mate pair sequencing. Within the 96 cases assayed by all three techniques, 23 (23%) cases

showed *PTEN* alterations by mate pair sequencing, compared with 29 (30%) and 29 (30%), by fluorescence *in situ* hybridization and immunohistochemistry, respectively. In the cases where *PTEN* loss was detected by mate pair sequencing confirmatory loss by both fluorescence *in situ* hybridization and immunohistochemistry was 100%. The additional cases detected by immunohistochemistry and fluorescence *in situ* hybridization were predominantly in the insignificant Gleason score 6 and large volume Gleason score 6 groups (Figure 3d). Of the 15 cases that showed *PTEN* alterations by fluorescence *in situ* hybridization or immunohistochemistry not detected by mate pair sequencing, ten were Gleason score 6 and just five showed loss by both immunohistochemistry and fluorescence *in situ* hybridization. The status of each case determined by each technique is presented in Figure 3e along with hemizygous or homozygous loss of *PTEN*.

***PTEN* Loss and Biochemical Recurrence**

Survival curves contrasting *PTEN* deletion detected by mate pair sequencing with biochemical recurrence as an end point demonstrated significance correlation (Figure 4a). Nearly 60% of patients with *PTEN* deletions by mate pair sequencing had a biochemical recurrence, compared with ~25% of patients without a deletion. In patients with Gleason score 7 and higher tumors, *PTEN* loss remained predictive of biochemical recurrence with 80% of patients whose tumors contained *PTEN* loss developing biochemical recurrence compared with 55% of patients whose tumor had intact *PTEN* (Figure 4b). There was no significant difference in the association of *PTEN* loss and biochemical recurrence by the three techniques (Supplementary Figure 3). The significance of *TMPRSS2-ERG* fusion status was also investigated (Figure 4c). Although the sample is small, a worse prognosis occurred in patients with that had a *TMPRSS2-ERG* fusion and loss of *PTEN*.

Discussion

Loss of the tumor suppressor, *PTEN*, has long been associated with high-grade prostate cancer. However, little is known about the frequency and impact of *PTEN* deletions and mutation in clinically insignificant prostate cancer. In this study we used a whole-genome sequencing approach to detect the genomic breakpoints in 126 prostate cancers treated by radical prostatectomy consisting of insignificant and large volume Gleason score 6, Gleason score 7 and 8 and higher grade cancers and compared sequencing results to fluorescence *in situ* hybridization and immunohistochemistry. The whole-genome mate pair sequencing approach enabled detection of all large genomic rearrangements impacting *PTEN*, and provided data to construct fluorescence *in situ*

hybridization probes to cover the majority of alterations. The results demonstrate that *PTEN* loss by mate pair sequencing, and fluorescence *in situ* hybridization and protein loss by immunohistochemistry is low in clinically insignificant prostate cancer, and loss (including homozygous loss) increases with increasing Gleason score. In addition, we did not identify any *PTEN* point mutations in clinically insignificant prostate cancer, and loss occurred in higher grade tumors associated with *PTEN* structural alterations resulting in homozygous *PTEN* loss. Finally, in Gleason score 7 cancer with *PTEN* alterations, the *PTEN* loss was detected in both Gleason pattern 3 and Gleason pattern 4 in 57% of cases by mate pair sequencing, 75% by fluorescence *in situ* hybridization, and 86% by immunohistochemistry.

In contrast to the well-characterized *TMPRSS2-ERG* fusion, our data indicates that rearrangements resulting in *PTEN* loss are highly heterogeneous between and within tumors (Table 2, Figure 2 and Supplementary Figure 2). Rearrangements were observed that spanned *PTEN*, or occurred within the 10q23 locus adjacent to or directly within the *PTEN* gene, with minimal similarity between cases. In addition to rearrangements solely within chromosome 10, many interchromosomal rearrangements were observed adjacent to or directly within *PTEN*, although no commonality was seen in partnering chromosomes between tumors. Complex events involving both multiple intra- and inter-chromosome 10 rearrangement events were also common resulting in genomic restructuring that was difficult to interpret but copy number variation analysis of mate pair sequencing data achieved by using frequency read-coverage from concordantly mapping mate pairs, confirmed loss of *PTEN*. Homozygous *PTEN* loss was also frequent and more common in the Gleason score 8 and higher tumors. Progressive losses were often observed in the Gleason score 7 group, where shared single loss events were evident between the Gleason pattern 3 and Gleason pattern 4, but with additional progressive losses within Gleason pattern 4 (Supplementary Figure 2 and Table 2). In no case, did we observe that chromothripsis or chromoplexis was a mechanism of *PTEN* loss.

PTEN loss was detected in 23% of cases by mate pair sequencing, and 31% of cases by fluorescence *in situ* hybridization and protein loss in 33% of cases by immunohistochemistry. In clinically insignificant cancer, loss was detected in one, four and two cases by mate pair sequencing, fluorescence *in situ* hybridization and IHC, respectively. However, only one of the four cases detected by fluorescence *in situ* hybridization demonstrated a loss of *PTEN* expression in the immunohistochemistry. Interestingly, the clinically insignificant cancer detected for *PTEN* loss by mate pair sequencing, was also detected by *in situ* hybridization, but no loss of *PTEN* expression was observed by immunohistochemistry. The higher detection of *PTEN* alterations by *in situ* hybridization

Figure 4 Survival curves of association of biochemical recurrence with *PTEN* loss. (a) *PTEN* loss from mate pair sequencing and biochemical recurrence in all cases. Case grouped according to *PTEN* normal (gray) or loss (black). (b) *PTEN* loss from mate pair sequencing data and biochemical recurrence in Gleason score 7 and above cases. Case grouped according to *PTEN* normal (gray) or loss (black). (c) Stratification of *PTEN* loss and *TMPRSS2-ERG* fusion (*ERG*) with biochemical recurrence. Case grouped according to *PTEN* loss/*ERG* positive (black solid) *PTEN* loss/*ERG* negative (black hashed) *PTEN* normal/*ERG* positive (gray solid) *PTEN* normal/*ERG* negative (gray hashed).

and loss of protein by immunohistochemistry can be explained by differences in the three techniques. Mate pair sequencing used small samples of pure population of tumor cells obtained by laser capture microdissection of frozen tissues compared with whole-section visual analysis by *in situ* hybridization and immunohistochemistry. Assessment of whole

sections allowed for the detection of populations of tumor cells that had *PTEN* loss that were likely missed by laser capture microdissection. However, the three techniques showed high concordance with infrequent loss of *PTEN* and protein in insignificant cancer, and a significant association with higher Gleason score and patient outcome. Comparison with

other studies is difficult because of technical differences. In our study, we used whole tumor sections rather than tissue microarrays, and developed specific probes based on the whole-genome mate pair sequencing data. Studies have shown a concordance of fluorescence *in situ* hybridization and immunohistochemistry for *PTEN* and protein loss but lower than our study. For instance, Lotan *et al* found that 45% of tumors that showed *PTEN* loss by immunohistochemistry had intact *PTEN* by fluorescence *in situ* hybridization.³³ This discordance can be explained by the complexity of the *PTEN* alterations. The heterogeneity of *PTEN* alterations in prostate cancer was appreciated by Reid *et al* who developed a probe strategy similar to our own with probes overlying the 3' and 5' as well as a third probe overlying the *PTEN* gene.³⁴ We confirm their hypothesis that gross rearrangements exist that cause disruption of *PTEN* in the absence of genomic deletion, and that a probe strategy that encompasses these larger rearrangements shows a higher concordance with immunohistochemistry, and explains the absence of *PTEN* protein in the setting of an intact *PTEN* as assessed by a single break-apart fluorescence *in situ* hybridization probe. Interestingly, in our study, both fluorescence *in situ* hybridization and immunohistochemistry demonstrated that in Gleason score 7 cancer the Gleason pattern 3 and 4 shared *PTEN* alterations and loss of protein in 75 and 86% of cases, respectively. This finding has significant implications in the interpretation of *PTEN* status in needle biopsy specimens. Recently, Lotan *et al* compared patients with Gleason score 6 on the needle biopsy specimen that had an upgrading of Gleason score in the radical prostatectomy specimen to patients that had Gleason score 6 in the needle biopsy and radical prostatectomy specimen.²³ They found that *PTEN* protein loss in the needle biopsy specimen was significantly associated with upgrading in the radical prostatectomy specimen. This is an important finding particularly in patients who are candidates of active surveillance where detection of a *PTEN* alteration on needle biopsy likely indicates the presence of clinically significant prostate cancer. One important limitation in assessing *PTEN* abnormalities in needle biopsy specimens includes the heterogeneity of *PTEN* loss in prostate cancers, and the limited sampling by biopsy. Indeed, although we did not specifically address the heterogeneity of *PTEN* alterations by fluorescence *in situ* hybridization, we did see heterogeneity in staining by immunohistochemistry. Although, nearly two-thirds of cases showed >50% of tumor staining loss, a third showed <50% staining loss. This heterogeneity will certainly impact the detection of *PTEN* alterations and lead to a significant false negative rate. However, we believe the detection of a *PTEN* alteration will remain clinically useful, and indicate the likelihood of clinically significant prostate cancer. We are currently applying our *PTEN* fluorescence *in situ* hybridization probe set to a large

cohort of matched needle biopsy and radical prostatectomy specimens to address the issues of heterogeneity, and sensitivity and false negative rates.

To the best of our knowledge, this is the first study to assess *PTEN* point mutations in clinically insignificant prostate cancer and high-grade prostatic intraepithelial neoplasia, and mutations were not identified in either. Although a less common pathway for *PTEN* loss, point mutations in the *PTEN* gene is important as a pathway for homozygous loss of *PTEN*. Six of 126 cases contained deleterious mutations, all in the Gleason score 7 and higher cancers. Mutations were generally late, occurring predominantly in the Gleason pattern 4 and 5. In contrast to *PTEN* structural alterations, only one case of Gleason score 7 shared a point mutation within Gleason pattern 3 and 4. In addition, these *PTEN* mutations all occurred in cases with additional *PTEN* deletions indicating a mechanism of achieving homozygous loss of *PTEN* function. Although not investigated in this study, epigenetic silencing of *PTEN* has been described as a further mechanism of *PTEN* expression loss, which could also function in association with mutations to drive homozygous *PTEN* loss.^{6,35} Haploinsufficiency of *PTEN* has been extensively associated with aggressive prostate cancer phenotype.^{9–10,17,36–40} Cells with haploinsufficiency of *PTEN* have been reported to be more unstable, defining a role for *PTEN* in maintaining chromosomal stability, as well increasing PI3K/AKT upregulation.^{36–41}

Although *PTEN* loss in prostate cancer has been reported as a progressive later event in prostate cancer, contradictory literature exists on the occurrence in high-grade prostatic intraepithelial neoplasia.^{10,17,23} In this study *PTEN* loss was assessed in 30 patients with high-grade prostatic intraepithelial neoplasia adjacent to the prostate cancer by whole-genome mate pair sequencing, Sanger sequencing, fluorescence *in situ* hybridization and immunohistochemistry. We identified no loss of *PTEN* expression, or any *PTEN* structural variations or point mutation in the high-grade prostatic intraepithelial neoplasia. This confirms recent reports, and suggests that *PTEN* alterations reported in high-grade prostatic intraepithelial neoplasia may be occurring in intraductal carcinoma.²³

In summary, whole-genome mate pair sequencing identified the heterogeneous and complex rearrangements resulting in *PTEN* loss in prostate cancer across the disease spectrum. *PTEN* deletion by whole-genome mate pair sequencing, fluorescence *in situ* hybridization and *PTEN* immunohistochemistry is infrequent in clinically insignificant prostate cancer and significantly correlated with increasing Gleason score and biochemical recurrence. Correlation between the three techniques was high. Fluorescence *in situ* hybridization and immunohistochemistry detected additional cases with *PTEN* loss, indicating a higher false negative rate by the whole-genome technique due to sampling.

In addition, using an *in situ* hybridization probe strategy that encompasses the spectrum of large chromosomal alterations detects higher frequency of tumors with *PTEN* deletions. Currently in our practice, we have implemented both the *PTEN* fluorescence *in situ* hybridization assay and immunohistochemistry. The *PTEN in situ* hybridization assay is performed in a cytogenetics laboratory with cytotechnologists dedicated to the assessment of clinical *in situ* hybridization assays. The *in situ* hybridization assay uses breakapart probes, and we believe can be readily implemented in clinical laboratories routinely performing clinical assays. It is important to recognize that small deletions can result in a diminished but not completely absent probe signal. We are currently in the process of comparing both assays on needle biopsy specimens. Whole-genome mate pair sequencing requires fresh-frozen tissue and is not applicable to biopsy specimens. Finally, detection of *PTEN* loss in Gleason score 6 in a needle biopsy specimen indicates a higher likelihood of clinically significant prostate cancer.

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Disclosure/conflict of interest

The authors declare no conflicts of interest.

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