BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Single-Cell Genetic Analysis Reveals Insights into Clonal Development of Prostate Cancers and Indicates Loss of PTEN as a Marker of Poor Prognosis

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Gauging the risk of developing progressive disease is a major challenge in prostate cancer patient management. We used genetic markers to understand genomic alteration dynamics during disease progression. By using a novel, advanced, multicolor fluorescence in situ hybridization approach, we enumerated copy numbers of six genes previously identified by array comparative genomic hybridization to be involved in aggressive prostate cancer [TBL1XR1, CTTNB2, MYC (alias c-myc), PTEN, MEN1, and PDGFB] in six nonrecurrent and seven recurrent radical prostatectomy cases. An ERG break-apart probe to detect TMPRSS2-ERG fusions was included. Subsequent hybridization of probe panels and cell relocation resulted in signal counts for all probes in each individual cell analyzed. Differences in the degree of chromosomal and genomic instability (ie, tumor heterogeneity) or the percentage of cells with TMPRSS2-ERG fusion between samples with or without progression were not observed. Tumors from patients that progressed had more chromosomal gains and losses, and showed a higher degree of selection for a predominant clonal pattern. PTEN loss was the most frequent aberration in progressors (57%), followed by TBL1XR1 gain (29%). MYC gain was observed in one progressor, which was the only lesion with an ERG gain, but no TMPRSS2-ERG fusion. According to our results, a probe set consisting of PTEN, MYC, and TBL1XR1 would detect progressers with 86% sensitivity and 100% specificity. This will be evaluated further in larger studies. (Am J Pathol 2014, 184: 2671–2686; http://dx.doi.org/10.1016/j.ajpath.2014.06.030)

Prostate cancer is the most commonly diagnosed noncutaneous neoplasm among US males (238,590 estimated cases in 2013) and is the second leading cause of cancer-related deaths (29,720 estimated deaths). Disease incidence exceeds mortality by a factor of 8; this suggests that many prostate cancers do not result in disease-associated death. This observation is attributable to the fact that many prostate cancers do not progress to metastatic disease. Patients with more indolent tumors would benefit from an active surveillance approach. Men with aggressive disease, however,
need immediate and often adjuvant therapy after radical prostatectomy (RP) to improve survival. Although serum-level screening for prostate-specific antigen (PSA) has increased detection of prostate cancer at earlier stages, sensitive and specific tests to distinguish men with indolent disease from men with aggressive prostate cancers are still lacking, which generates a dilemma in how to adapt risk-associated treatments.

Numerous studies have identified genomic changes as potential predictors of progression. Perhaps the best-known tumor marker specific to prostate cancer is the fusion of TMPRSS2 and ERG on chromosome 21. Fusions of these two genes have been observed in approximately 30% to 60% of prostate cancers, but whether the gene fusion predicts tumor progression is controversial. One explanation might be that many possible TMPRSS2-ERG fusions exist, which result in transcripts with different consequences for disease prognostication. An additional problem in elucidating the role of TMPRSS2-ERG in tumor progression might be intratumor heterogeneity.

Deep sequencing of somatic mutations and approaches to enumerate copy number variation on the level of single cells in cancer have led to increasing recognition of the importance of such intratumor heterogeneity in cancer progression. Herein, we explore intratumor heterogeneity of prostate tumors using a special break-apart probe for the TMPRSS2-ERG fusion and six single-gene probes selected on the basis of a prior array comparative genome hybridization (aCGH) study.

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As for MEN1, whether TBLIXRI functions as an oncogene or as a tumor suppressor varies by tumor type. TBLIXRI is overexpressed and in a region of recurrent copy number gains in lung cancer and in breast cancer. However, TBLIXRI is recurrently mutated or deleted in hematological malignancies, and its expression inhibits the growth of head and neck cancer cells.

Platelet-derived growth factor (PDGF) comprises a set of four ligands (PDGFs A, B, C, and D) that bind to two receptors (PDGFRα and PDGFRβ) to deliver signals that affect cell growth, cell shape, and chemotaxis. PDGFRα and PDGFRβ, which encode the receptor units, are overexpressed in bone marrow metastases of prostate cancer, and higher PDGFRβ expression is part of a five-gene expression-based predictor for prostate cancer recurrence. There is a competition between two of its ligands, PDGFB and PDGFβD, in prostate cancer, such that when PDEN is lost, PDGFβD is preferred.

CTTNBP2 influences the size and number of dendritic spines. CTTNBP2 has roles in other tissues, including binding of the RAD21-cohesin complex. More specific to prostate cancer, CTTNBP2 is in a block of genes on chromosome 7 that is differentially methylated in prostate cancer cell lines.

We applied two layers of analysis to this panel of FISH probes. First, we pursued the conventional strategy of profiling the probe set in subsets of tumor samples from RP patients with different clinical outcomes (herein, non-
progressers and progressers) to identify combinations of probes that distinguish patient groups. We also pursued a novel, unconventional goal of building a mathematical model of cellular-level progression for each tumor sample and derived test statistics that can collectively distinguish the two groups of patients.38 The first goal is fulfilled with a candidate three-probe test, which effectively distinguishes progressers from non-progressers in our study; however, it will require validation in a larger sample. Independent of that, our modeling work provided insights into genome dynamics of prostate cancer progression.

**Materials and Methods**

**Materials**

FFPE surgery specimens of prostate carcinomas from six patients with non-progressive disease and seven patients with progressive disease after RP were retrieved from the archives of the Tissue Core at the Helen Diller Family Comprehensive Cancer Center, University of California (San Francisco, CA), using samples designated for research purposes. Recurrence or progressive disease was defined as a secondary PSA measurement within 1 year of ≥0.2 ng/mL and/or evidence of metastatic disease. The clinical data are summarized in Table 1.

The material was prepared using a dividing procedure that has been described previously.31 The hematoxylin and eosin (H&E)—stained sections were used to verify that each section consisted of at least 50% tumor material. The 50-μm unstained sections were then disintegrated, and cytospins were prepared as described.31

In addition, we used three FFPE surgery specimens of prostate carcinomas from the University Medical Center Schleswig-Holstein, Campus Lübeck (Lübeck, Germany), to test a three-color FISH probe panel on tissue sections (4 μm thick) to assess the feasibility of implementing this test in routine pathological analysis.

The material for the current study has been coded, and an exemption has been issued by the NIH Office of Human Subjects Research for use of the de-identified data.

**FISH Data**

The cytopsins were evaluated by FISH with CEPs for centromeres 8 and 10 and six locus-specific identifier probes for the following genes: TBLIXRI (3q26.23), CTTNB2P2 (7q31.2), MYC (8q24.21), PTEN (10q23.1), MEN1 (11q13), and PDGFB (22q13.1) (Abbott Molecular, Des Plaines, IL). The probes were selected on the basis of previous aCGH data,32 and each probe represented approximately 200 to 600 kb of genomic sequence centered on the gene of interest; the probes were directly labeled with fluorophores. The centromere probes, PTEN and MYC, were labeled in SpectrumAqua or SpectrumGreen (Abbott Molecular). CTTNB2P2 and MEN1 were labeled in SpectrumRed, whereas PDGFB and TBLIXRI were labeled in SpectrumGold. The FISH probes were combined into two panels. The panel for the first hybridization consisted of CEP10, PTEN, CTTNB2P2, and PDGFB. The panel for the second, subsequent hybridization contained CEP8, MYC, MEN1, and TBLIXRI. The samples were also evaluated for their TMPRSS2-ERG fusion status with an ERG break-apart probe10 in a third, subsequent hybridization. The ERG probe hybridization was recorded in three-digit patterns for each cell, with the first digit registering the number of normal ERG alleles (red and green signals on top of each other), the second digit referring to the number of telomeric ERG probe signals (single green signals), and the third digit representing the number of centromeric ERG probe signals (single red signals). Therefore, a 200 pattern would be indicative of a normal diploid status of ERG, whereas a 111

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**Table 1  Clinical Patient Data**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Patient no.</th>
<th>Age (years)</th>
<th>PreOp PSA (ng/mL)</th>
<th>Gleason primary</th>
<th>Gleason secondary</th>
<th>Gleason sum</th>
<th>Surgical year</th>
<th>Follow-up duration or time to recur (months)</th>
<th>Margin status</th>
<th>Seminal vesicle involvement</th>
<th>Lymph node involvement</th>
<th>Extracapsular extension</th>
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<td>7.2</td>
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<td>5</td>
<td>8</td>
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<td>72.16</td>
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<td>No</td>
<td>Neg</td>
<td>No</td>
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<tr>
<td></td>
<td>2</td>
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<td>4</td>
<td>7</td>
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<td>30.81</td>
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<td>No</td>
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<td>3</td>
<td>4</td>
<td>7</td>
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<tr>
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<td>4</td>
<td>52.5</td>
<td>5.1</td>
<td>3</td>
<td>5</td>
<td>8</td>
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<td>4</td>
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<td>13</td>
<td>60</td>
<td>24</td>
<td>4</td>
<td>3</td>
<td>7</td>
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<td>1.84</td>
<td>Neg</td>
<td>Yes</td>
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</tbody>
</table>

Neg, negative; Pos, positive; PreOp, preoperative.
or a 101 pattern would be indicative of a fusion of TMPRSS2-ERG by insertion/translocation or deletion, respectively.10

One cytospin per case was hybridized and evaluated with the above mentioned three different probe sets using single-cell FISH methods, as described previously.31 Detection of the ERG break-apart probe was performed according to Perner et al.10 In all 13 samples, all probes could be counted. This resulted in an eight-probe signal pattern plus a three-digit TMPRSS2-ERG pattern for each cell analyzed, with an average of 316 (range, 197 to 372) interphase nuclei counted for each case. As previously described,31 for each cell, a ploidy value (diploid, triploid, or tetraploid) was assigned to each signal pattern on the basis of the assessment of the pattern. A computer program developed to assign gain and loss patterns by comparing signal counts to the ploidy value59 was applied, and the patterns were sorted according to their frequency and displayed in color charts giving an overview of the clonal populations and the overall heterogeneity observed in the tumors (Figure 1).31

The tissue sections (4 μm thick) were deparaffinized in xylene, hydrated, and pretreated with 0.02% pepsin for 25 to 70 minutes. Slides were then washed in 1× phosphate-buffered saline, dehydrated, air dried, and codenatured at 72°C for 5 minutes with a three-color probe panel consisting of MYC-TBL1XR1-PTEN. The probe panel was either labeled in-house by nick translation using Dynomics 415-dUTP (Dynomics, Jena, Germany) for MYC or Dynomics 505-dUTP for TBL1XR1 and SpectrumOrange-dUTP (Abbott Molecular) for PTEN, or provided by Abbott Molecular in the following color scheme: MYC in SpectrumAqua, PTEN in SpectrumGreen, and TBL1XR1 in SpectrumGold. The slides were detected with a 2-minute wash in 2× standard saline citrate (SSC)/0.3% NP40 at 48°C, followed by 1 minute in 2× SSC/0.1% NP40 and 1 minute in 2× SSC, both at room temperature. Slides were air dried and coverslipped, and images were taken with a Leica DMRXA microscope (Leica, Wetzlar, Germany) equipped with custom filters (Chroma, Bellows Falls, VT) and a CoolSnap camera (Photometrics, Tucson, AZ).

DNA Ploidy Measurements

Nuclear DNA ploidy status was assessed by image cytometry using Feulgen-stained cytospins of dissociated cells from all samples, except for case 10, because of insufficient cell material. The staining procedure, internal standardization, and cell selection criteria were based on published methods.60 At least 7803 particles per sample (mean, 24,000; range, 7803 to 72,101) were detected automatically using the ICM imaging system (Ahrens ICM Cytometry System; Meßtechnische Beratung, Bargteheide/Hamburg, Germany). At least 603 cells (mean, 2953; range, 603 to 8422) per sample were interactively selected in the ICM cell gallery and quantitatively measured for their DNA content. All DNA values were expressed in relation to the corresponding staining controls (lymphocytes), which were given the value 2c, denoting normal diploid DNA content. The DNA profiles were classified as previously described.60 Samples were assessed as euploid when <5% of cells showed DNA values >4.5c. Aneuploidy was defined as >5% of cells presenting with DNA values exceeding the tetraploid region (>4.5c).

aCGH Data

The previously acquired aCGH data for regions containing the genes used in our FISH analysis were extracted from the existing data set.61 The aCGH-based copy number calls for all 13 cases and 6 genes were recorded and aligned with the corresponding FISH calls for the purpose of comparing by correlation analysis of the copy number estimates obtained by aCGH and by FISH.

Analysis of Tumor Heterogeneity

We refer to the ordered list of count values for each probe as a signal count pattern. To explore the possibility that tumors with progression are more or less heterogeneous than tumors without progression, we compared three measures of diversity in the distribution of FISH signal count patterns: i) instability index, ii) Shannon index, and iii) Simpson index.62 The indices were computed for each sample and were then compared between the progresser and non-progresser distributions by either comparing the mean or using a Wilcoxon signed-rank test. The instability index is defined as 100 times the number of cell count patterns divided by the number of cells. To define the other two indices, let \( p_i \) be the probability of the \( i \)th cell count pattern. Then, the Shannon index, which is commonly used in information theory (alias entropy), is as follows:

\[
-S \sum p_i \log_2(p_i).
\]  

The Simpson index, which is commonly used in population genetics, is \( \Sigma p_i^2 \).

Estimation of Empirical Significance of a Set of Gene Gains/Losses to Distinguish Non-Progressers versus Progressers

To assign an empirical \( P \) value for sets of gains and losses that may distinguish non-progressers versus progressors, we used simulation. Permutation tests were performed in which a status of gain/loss/neutral for each of the six genes (TBL1XR1, CTTNBP2, MYC, PTEN, MEN1, and PDGFb) was assigned at random to 13 samples, with the progresser/non-progresser status permuted. In each replicate, the number of samples with a gain or loss of each gene was fixed to match the observed data. In this context, a classifier is a nonempty set of gene gains and losses from the six genes that predicts whether a sample comes from a progresser, if at least one of the gains and losses is present, or from a non-
progressor, if at least one of the specified gains and losses is not present. Each gene may be specified as gained, specified as lost, or not used in the classifier. Therefore, there are 729 possible classifiers ($3^6 - 1$). For each replicate $r$, we considered all possible classifiers. The test statistic $A$ (classifier, $r$) assigned to a classifier was the arithmetic mean of sensitivity and specificity. For each of the 10,000 replicates $r$, the test statistic $S(r)$ was the maximum test statistic $A$ among all possible classifiers for fixed $r$. The empirical $P$ value assigned to a classifier of the observed data was the fraction of replicates $r$ for which the $S(r)$ is greater than or equal to $A$ (classifier, observed data).

Modeling Tumor Progression and Analysis of Node Depth

For each of the 13 tumors, we modeled the progression of copy number changes using FISHtrees software, which infers phylogenetic trees describing progression among the observed cell types as distinguished by their probe copy numbers. A tree is inferred from the data for each tumor to heuristically seek to minimize the total number of copy number changes across the tree. In this analysis, we used the six gene probes and we used the break-apart probe to assess the copy number of ERG. We did not use the fusion status or the centromere probes. For each tumor, FISHtrees generated a tree model in which the normal state (2, 2, 2, 2, 2, 2) is at the root of the inferred tree and each edge moving away from the root to a new node corresponds to a change in copy number of one gene. For each node, we also stored the number of cells observed to match the seven-component signal count pattern for that node.

The number of steps away from the root is usually called the depth of a node. Our prior work on cervical cancer progression trees showed that the distribution of cells by depths provides a measure of tree topology that is predictive of progression potential. To test whether this characterization of tree topology is similarly predictive of prostate cancer progression, we performed an analogous test. We computed the percentages of cells represented by nodes at each depth in the tree, as described previously. We used percentages of cells at each depth rather than total cells to normalize for differing numbers of cells analyzed for different tumors. We visualized the distribution of the depths of cells in progressors versus non-progressors by a bar graph. We then tested for significance of the difference between average depths for non-progressors versus progressors by a Wilcoxon signed-rank test. Because we hypothesized that the trees derived from progressor samples would have greater average depth, the Wilcoxon $P$ values were one sided.

Results

In a previously published aCGH study, we identified loci that were differentially gained or lost in primary prostate cancers from patients with non-progressive or progressive disease after RP. A subset of the genes was validated by TaqMan analysis. On the basis of these results and the feasibility of probe design, FISH probes centered on the genes of interest were selected. We hybridized the selected FISH probes targeting the six most promising markers [ie, TBLIX1R1 (3q26.23), CTNNBP2 (7q31.2), MYC (8q24.21), PTEN (10q23.1), MEN1 (11q13), and PDGFB (22q13.1)], together with centromere probes for chromosomes 8 and 10 and an ERG break-apart probe for determining whether the fusion status of TMPRSS2-ERG could serve as an additional progression marker. The probes were sequentially hybridized to interphase cells prepared as cytospins from 13 primary prostate carcinomas (six non-progressors and seven progressors). Signal patterns were counted in 197 to 372 nuclei per sample (average, 316 nuclei), excluding nuclei with two signals for all probes and a 200 pattern (indicating two normal alleles) for the ERG break-apart probe.

Clinical Features

Pertinent clinical parameters are summarized in Table 1. All patients were at high risk of recurrence by the D’Amico Risk Classification. No clinical variables were found to be confounding.

Chromosomal Instability and Clonal Patterns

The FISH probe panels were hybridized sequentially to individual nuclei of the same specimen. Repeated hybridization and relocation of the cells afforded us the possibility of enumerating clonal aberration patterns on a cell-to-cell basis for all six gene probes, the two centromere probes, and the ERG break-apart probe. The FISH signal patterns were assigned to two groups: patterns for which each cell fitting the pattern had an identical count for all signals, termed signal pattern clones, and patterns for which each cell fitting the pattern matched each other cell in the direction of change (gain, loss, or normal) of each signal but not necessarily in the exact counts, termed imbalance clones. For example, in case 10, the major imbalance clone is gain of TBLIX1R1, gain of CTNNBP2, normal for MYC, loss of PTEN, normal for MEN1, normal for PDGFB, and break-apart probe pattern 101 (case 10) (Figure 1). To visualize and compare major imbalance clones in prostate carcinomas with or without progressive disease, each cell of the lesion was displayed according to its ERG break-apart patterns and its gain, loss, or unchanged (normal) status, with the gene probes sorted according to their chromosomal location from the top to the bottom of the chart and with the patterns observed displayed from left to right sorted by frequency (Figure 1). In addition, the frequency of gained and lost status for each of the gene loci and each ERG break-apart pattern was calculated in percentages of the total cell population, the average ploidy of the lesion, and the average
Figure 1  Summary of imbalance clones in six cases of primary prostate carcinoma without progression (cases 1 to 6; A) and seven cases with progression (cases 7 to 13; B). Green, gains; red, losses; blue, unchanged. The organization of the graphs is the same for all cases and is explained for case 1 in detail from left to right. The Locus column shows the chromosome arm. Each vertical line separates the most common imbalance clones. The row above the imbalance clones displays the percentages at which the clones were found. For example, the most frequent clone in case 1 comprised 19.4% of the cells with an ERG break-apart fusion pattern of 111, indicating fusion by insertion, but exhibited no other gains or losses. The second most frequent clone comprised 9.0% of the cells and had the same ERG pattern 111 but showed, in addition to that change, a gain of MEN1. Two clones that each comprised 3.2% of the tumor cell population followed. Both showed also the 111 pattern for fusion, but one of them had a loss for PTEN and the other displayed a loss for MEN1. The Marker column shows the gene/probe name. The Gain column shows that 25% of the cells had a gain of MEN1. None of the gains and losses comprised >30% of the cell population in case 1. The Average sig. no. column shows that the average signal count for MEN1 in the entire population was 2.2. The percentage of gains or losses in >30% of the cells is in red (loss) and green (gain), respectively, highlighting (e.g., MEN1 gain in case 2). Two hundred seventy-eight nuclei were counted for case 1. Average ploidy values were calculated from the ploidy values assigned to each nucleus by signal patterns (as described in Materials and Methods). The numerical ERG break-apart patterns (as described in Materials and Methods) are displayed in the bottom row. Fusion events are yellow, and patterns indicating normal ERG alleles are light blue. The four most frequently observed ERG break-apart patterns are displayed at the bottom right.
The most frequent imbalance clone in each tumor comprised, on average, 20.8% of the cell population, with a range of 11% to 44% (Figure 1 and Table 2). Interestingly, the average size of the major imbalance clone in the progressors was 24.6% (average size for major signal pattern clone, 20.6%), whereas the average size in non-progressors was 16.5% (average size for major signal pattern clone, 14.0%).

### Table 2  Summary of Results for Primary Prostate Carcinomas with and without Progressive Disease

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Case no.</th>
<th>Total no. of cells counted</th>
<th>Total no. of signal patterns</th>
<th>Instability index (no. of patterns × 100/no. of cells)</th>
<th>% Diploid cells (according to FISH signals)</th>
<th>DNA ploidy measurement</th>
<th>Stem line</th>
<th>Major clonal imbalance patterns</th>
<th>Major clonal signal patterns</th>
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<td>Non-progressers</td>
<td>1</td>
<td>278</td>
<td>139</td>
<td>50</td>
<td>100.0</td>
<td>Euploid 1.92</td>
<td>80.2</td>
<td>19% Fusion, 9% Fusion + MEN1 gain</td>
<td>19% 22222222111</td>
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<td>2</td>
<td>382</td>
<td>226</td>
<td>59.2</td>
<td>70.9</td>
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<td>85</td>
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<td>208</td>
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<td>11% Fusion, 5% No fusion + MEN1 gain, 4% Fusion + MEN1 gain</td>
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<td>159</td>
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<td>28% 12222222111</td>
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<tr>
<td></td>
<td>6</td>
<td>257</td>
<td>139</td>
<td>54.1</td>
<td>77.8</td>
<td>Euploid 2.02</td>
<td>60.7</td>
<td>16% Fusion + CEPI8 loss, 5% No fusion + CTNBP22 loss, 4% Fusion + CEPI8 loss + CTNBP2 gain</td>
<td>11% 22222222101, 5% 24444444202</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>305.2</td>
<td>159.3</td>
<td>51.7</td>
<td>84.6</td>
<td>NA</td>
<td>65.5</td>
<td>Average size of major imbalance clone = 16.5%</td>
<td>Average size of major signal pattern clone = 14.0%</td>
</tr>
<tr>
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<td>7</td>
<td>330</td>
<td>212</td>
<td>64.2</td>
<td>88.7</td>
<td>Euploid 2</td>
<td>5.2</td>
<td>24% ERG gain + CEPI8 gain + MYC (alias c-myc) gain, 6% ERG gain + CEPI8 gain + MYC gain + MEN1 gain</td>
<td>9% 42225222300, 4% 32225222300, 3% 42224222300</td>
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<tr>
<td></td>
<td>8</td>
<td>372</td>
<td>96</td>
<td>25.8</td>
<td>97.6</td>
<td>Euploid 2.01</td>
<td>53.5</td>
<td>30% Fusion, 14% No fusion + CEPI8 loss, 9% No fusion + PDGFI loss</td>
<td>30% 22222222111</td>
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<td></td>
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<td>340</td>
<td>158</td>
<td>46.5</td>
<td>95.3</td>
<td>Euploid 2.01</td>
<td>84.1</td>
<td>15% Fusion, 11% Fusion + PTEN loss, 7% Fusion + CEPI10 gain + PTEN loss</td>
<td>15% 22222222111</td>
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<tr>
<td></td>
<td>10</td>
<td>278</td>
<td>157</td>
<td>56.5</td>
<td>96.4</td>
<td>ND ND</td>
<td>73.7</td>
<td>24% Fusion + TBL1XR1 gain + CTNBP2 gain + PTEN, 4% Fusion + CTNBP2 gain + PTEN loss</td>
<td>23% 22332022101</td>
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<tr>
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<td>325.4</td>
<td>147.9</td>
<td>46.6</td>
<td>96.0</td>
<td>NA</td>
<td>65.2</td>
<td>Average size of major imbalance clone = 24.6%</td>
<td>Average size of major signal pattern clone = 20.6%</td>
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<td>316.1</td>
<td>153.2</td>
<td>49</td>
<td>90.8</td>
<td>NA</td>
<td>NA</td>
<td>65.4</td>
<td>Average size of major clone = 20.8%</td>
<td>Average size of major signal pattern clone = 17.5%</td>
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</table>

NA, not applicable; ND, not determined.
was only 16.5% (14% for major signal pattern clone), suggesting that progressors have a higher degree of selection for a specific clone. However, there were only slight differences in the average instability index measured as the number of FISH signal patterns per 100 cells (46.6 versus 51.7) and the average percentage cells with fusion for TMPRSS2-ERG (65.2% versus 65.5%) between samples from patients with or without progression (Table 2). Wilcoxon tests of the instability index, Shannon index, and Simpson index comparing non-progressers and progressers showed no statistically significant differences in these measures of heterogeneity. Also, nuclear ploidy measurements could not discern between non-progressors and progressers.

The major clones of all lesions showed fusion events of TMPRSS2-ERG, except for one progressor, which was the only case that displayed gains of MYC and CEP8, indicating a gain of the entire chromosome 8 (case 7) (Figure 1). This was also the only case with gains of a normal ERG allele; therefore, it might follow a different pathway to progressive disease. Four progressers, cases 9, 10, 11, and 12 (Figure 1), revealed major clones with PTEN loss, which was not observed in any of the non-progressors. In addition, case 9 had a major clone with only a fusion event for TMPRSS2-ERG. The remaining two progressors had major clones with either only a fusion event (case 8) (Figure 1) or a fusion event with a TBL1XR1 gain (case 13) (Figure 1), respectively. The aberrations in the major clones of non-progressing tumors were fusion events only (cases 1, 3, and 4) (Figure 1), a fusion event with a MEN1 gain (case 2) (Figure 1), and TMPRSS2-ERG fusion events with a CEP8 loss (cases 5 and 6) (Figure 1).

Table 3 shows that overall more chromosomal gains and losses were found in carcinomas from the progressor group, whereas carcinomas of the non-progressing patients showed few changes, involving the loss of CEP8 and the gain of MEN1. Specifically, three gains/losses in the non-progressor group and 13 gains/losses in the progressor group are listed (Table 3). To evaluate whether the imbalance in the distribution is statistically significant, we permuted the entries in (a copy of) each column 10,000 times (Table 3). One can obtain an empirical one-sided P value by asking what proportion of the 10,000 scrambled replicates has, at most, three gains/losses in the non-progresser group and correspondingly at least 13 gains/losses in the progressor group. By using this permutation test, we obtained \( P = 0.0143 \).

**TMPRSS2-ERG Fusion**

In 12 of 13 lesions, the break-apart probe indicates clonal patterns with a TMPRSS2-ERG fusion. There was no significant difference in the average percentage of cells with fusion (65.2% versus 65.5%) between samples from patients with or without progression (Table 2). Most cases exhibited tumor cell populations that were heterogeneous for the ERG break-apart probe, often including a population with two normal alleles for ERG (pattern 200) (Figure 1). However, 6 of the 13 cases showed a specific fusion pattern in >60% of their tumor cell population, indicating that in those tumors either this was an early event in tumorigenesis or there was a strong selection for this particular pattern. There was no significant difference in the distribution of fusions by either insertion or translocation (pattern 111) and deletion (pattern 101) in the progressors and the non-progressors. Two non-progressors, but none of the progressors, revealed clonal cell populations with a double deletion of the sequences between ERG and TMPRSS2 (cases 4 and 6) (Figure 1). However, the double deletion pattern 202 was observed in tetraploid cells, indicating a duplication of a diploid cell with the single deletion pattern 101. There was one progressor with a gain of a normal ERG allele (case 7). This case was also the only case with a gain of MYC.

Various studies have suggested that ERG-TMPRSS2 fusions are early events in prostate cancer development.\(^{20,64,65}\) In our samples, there were five cases (cases 1, 5, 9, 10, and 13) (Figure 1) that revealed clones with the same ERG break-apart pattern but different gain and loss patterns for the other genes analyzed, indicating that the TMPRSS2-ERG fusion

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Case no.</th>
<th>CEP8</th>
<th>CEP10</th>
<th>TBL1XR1</th>
<th>CTNB1P2</th>
<th>MYC (alias c-myc)</th>
<th>PTEN</th>
<th>MEN1</th>
<th>PDGFB</th>
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<tr>
<td>Progressers</td>
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<tr>
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<td>11*</td>
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<td>13*</td>
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</table>

*Cases were detected by a three-probe panel consisting of FISH probes for PTEN, MYC, and TBL1XR1.
happened as the first event, followed by specific chromosomal gains and losses. However, four cases (cases 2, 7, 8, and 11) (Figure 1) showed clones with the same gain and loss patterns, but different ERG break-apart patterns, which might indicate that the cells first acquired a specific chromosomal imbalance, and that the TMPRSS2-ERG fusion happened as a later event. In the remaining four cases (cases 3, 4, 6, and 12) (Figure 1), there were different ERG break-apart patterns with different gain and loss patterns, most likely indicating a parallel development of different clones. This is surprising because it appeared more likely that within one tumor focus, fusion status should be clonal, although interfocal heterogeneity in the TMPRSS2-ERG fusions of multifocal prostate tumors had been reported.25 The 202 pattern in case 6 can be explained with a tetraploidization of the genome that resulted in a duplication of the 101 pattern seen in the major clone of this case.

Correlation of aCGH and FISH

The correlation between gains and losses called by aCGH and FISH was 83.3%61 when using a threshold of >30% of the cells with gain or loss for FISH. Most of the discrepant calls are due to CTTNB2 gains called by aCGH but not seen by FISH, and MEN1 gains seen by FISH but not called by aCGH.

DNA Ploidy Measurement Results

Image cytometry measuring the nuclear DNA content and determining the ploidy status did not reveal any aneuploid lesions among the six non-progresser and six of the seven progresser cases (one case did not have adequate material to be measured) (Table 2). All cases had diploid stem lines. A few cases showed small fractions of proliferating and/or tetraploid cells, which was in concordance with our FISH signal pattern observations (Table 2). In summary, all cases showed euploid DNA histograms indicating that DNA ploidy measurements were not able to discern non-progressers from progressers in our study.

Performance of the FISH Probes as Progression Markers

Figures 2 and 3 and Table 3 give a summary of the overall performance of the six gene markers and the two centromere probes tested with regard to differential gain and loss patterns in progressive and non-progressive prostate carcinomas. Figure 2 shows the average gain and loss frequencies for all of the gene markers and two centromere probes observed in all prostate carcinoma cells analyzed, grouped by patients with or without progression. The most frequent change observed affects PTEN in the progressive disease group with a loss in >40% of the cells, compared with only 11% of the non-progresser group. The progresser group showed, on average, a 10% to 15% higher percentage of cells with TBL1XR1, CTTNB2, or MYC gain compared with the non-progressers. However, MEN1 and PDGFB showed similar percentages for progressers and non-progressers. In fact, MEN1 gain was more frequently observed in the cells of non-progressing carcinomas.

When using a threshold of >30% of the cells showing the aberration, lesions from patients with progressive disease had an average of 1.9 gains/losses for the probes tested, compared with a substantially lower average of 0.5 for tumors from patients without progression (Figure 3). By using the same threshold, the loss of PTEN was the most frequent aberration in the progressers (4/7 = 57%), and was not observed in non-progressers, confirming its potential as a marker for aggressive disease. TBL1XR1 was gained in two of the seven progressers (29%), whereas none of the non-progressers showed changes in the copy number of this gene. One progresser had a gain of CTTNB2 and another progresser had a loss of this gene, whereas none of the non-progressers showed

![Figure 2](image_url) Average gain and loss frequencies for all of the gene markers and two centromere probes observed in prostate carcinoma cells of patients with and without progression. Percentages of cells with gains are shown above the 0% line and with losses below the 0% line.

![Figure 3](image_url) Specific gains and losses observed in non-progressing and progressive prostate cancer disease. CEPB and CTTNB2 were gained and lost. The thicknesses of the arrows reflect the percentage of change from non-progressive to progressive disease. The increase of lesions with a loss of PTEN is the most pronounced.
CTTNBP2 copy number changes. One progresser revealed CEP8 and MYC gains, indicative of a chromosome 8 gain. The gain of MYC was not observed in non-progressers. MEN1 was gained in one progresser and one non-progresser lesion, whereas PDGFB was not changed in any of the lesions, indicating that these two genes are not differentially gained or lost in progressors. A probe set consisting of PTEN, MYC, and TBL1XR1 would have detected six of the seven progressers analyzed, which is equivalent to a test sensitivity of 86% (Table 3). The one progresser case that would not have been detected (case 8) had a major clone (30% of the cell population) with a TMPRSS2-ERG fusion pattern of 111 and two minor clones, one comprising 12% of the cell population with an unusual ERG loss and a loss of CEP8 indicating an 8p loss and another clone comprising approximately 10% of the cell population revealing a rare loss of PDGFB on a normal diploid background without a TMPRSS2-ERG fusion (Figure 3). All non-progressers would have been negative for a test with a probe set of PTEN, MYC, and TBL1XR1 (100% specificity), indicating that the combination of these three markers might have potential to predict progression in prostate carcinomas. By using the permutation test method described above, the combination of 86% sensitivity and 100% specificity has an empirical P value of 0.013.

Applying the MYC-TBL1XR1-PTEN Probe Panel to Tissue Sections of Prostate Cancers

We tested whether the three-color FISH probe panel that was determined to have the highest combined sensitivity and specificity (namely, MYC-TBL1XR1-PTEN) can be easily applied to routine tissue sections of FFPE material. We successfully hybridized three different prostate cancer specimens. The hybridization of one of the prostate cancer cases and the H&E stain for the tumor is shown in Figure 4. The tumor cells did not show any PTEN signal, indicative of a biallelic loss of PTEN (Figure 4B), whereas normal ducts in the neighboring tissue revealed a normal signal pattern with mostly two signals per nucleus and probe. Signal counts of fewer than two are more likely due to the truncation of cells (Figure 4A). TBL1XR1 and MYC revealed mostly four signals per tumor nucleus, indicating a possible gain for both markers in the tumor cells (Figure 4B). We also observed truncation artifacts due to sectioning.

Tree Models of Tumor Progression Show a Different Pattern of Changes in Non-Progressers versus Progressers

Although, in this study, the instability indexes of the prostate carcinomas with or without progression after RP are similar, the frequency of genomic imbalances is substantially higher in the progresser group, with 1.9 gains or losses per case versus 0.5 gains or losses in the non-progressers. To visualize the pattern of progression in each case, we constructed tree models of progression using FISHTrees software, which infers phylogenetic trees describing likely evolution of the set

Figure 4 FISH on tissue sections with a prostate cancer-specific FISH probe panel. A: Normal prostate; most cells reveal two signals for the three probes [MYC (alias c-myc) in red, TBL1XR1 in green, and PTEN in yellow]. Some cells are truncated because of sectioning. B: Prostate cancer; four copies for the probes MYC (red signals) and TBL1XR1 (green signals) are present in most cells; again, cells are often truncated because of sectioning. We did not observe signals for the PTEN-specific probe, consistent with a biallelic deletion of this gene. C: Representative area of H&E-stained section of this cancer.

Figure 5 Distribution of cells across different levels of tumor progression trees for non-progresser (light gray bars) and progresser (dark gray bars) cases.
of observed signal counts within each tumor from an initially diploid root cell to heuristically minimize total copy number changes across the tree (Supplemental Figures S1–S13).  
To evaluate whether there were statistically significant differences between the inferred phylogenetic trees of non-progressers versus progressers, the cell distribution across tree levels was calculated (Figure 5). The analysis showed that in the non-progressers, 70% of all cells were distributed within the first three tree levels, which was true for only 44% of the cells of the lesions that progressed. This observation indicates that cells of lesions that have a higher propensity to progress to advanced disease, on average, deviate more from the normal diploid status compared with cells from non-progressing lesions. This observation can be formalized statistically by computing weighted average depth of the nodes up to some level $L$ for the six non-progressers and the seven progressers. The weighted average depths for $L = 5,...,12$ for the two sets (non-progressers versus progressers) were compared by a Wilcoxon signed-rank test, which shows that the weighted average depth in the progressers is statistically significantly greater (Supplemental Table S1). For example, for $L = 10$, the $P$ value of the test is 0.018. The node depth is the distance away from the normal signal count pattern $(2,...,2)$ expressed in terms of the count of copy number changes. Thus, the cells in the progresser samples have in general a trend toward more total chromosomal changes. This trend is not captured by previously proposed measures of diversity (Shannon or Simpson index).

**Discussion**

Men with slowly progressing prostate cancers could be treated with active surveillance approaches instead of immediate, more aggressive treatment, including surgery, which can have considerable adverse effects. This subset of patients will become larger as populations age and more tumors are detected early by screening efforts. Distinguishing patients with aggressive or indolent prostate carcinomas would help in designing risk-adapted neoadjuvant and adjuvant treatments.

Herein, we used single-cell genetic analysis of copy number changes and chromosomal translocations on the basis of interphase cytogenetics (FISH) to understand genome dynamics in prostate tumors from patients with or without progression after RP. This allowed us to evaluate a set of genetic markers for their usefulness to predict progression, identify pathways of carcinogenesis, and examine patterns of genomic imbalances and clonal evolution. We used three FISH probe panels targeting six genes identified by aCGH to be differentially gained and lost in tumors that progressed compared with tumors that did not progress. In addition, the TMPRSS2-ERG fusion status was assessed in the same cells with a FISH ERG break-apart probe, and two centromere probes were hybridized as control probes. The sequential hybridization of these panels to intact nuclei prepared from prostate carcinomas enabled us to enumerate all probe counts in multiple individual cells.

The non-progressing prostate carcinomas revealed a slightly higher average chromosomal instability index, calculated as the number of distinct signal patterns per 100 cells, with an average of 51.7 patterns (range, 41.7 to 59.2), compared with the progressing lesions, with an average of 46.6 patterns (range, 23.8 to 64.2). The range in the non-progressing lesions was narrower because two progresser lesions showed substantially lower indices (23.8 and 25.8) than the rest of the group (46.5 to 64.2), reflecting the fact that these two lesions had large populations of a major stable clone comprising 44% and 30% of the total tumor population. We have previously observed a higher average chromosomal instability index in a multicolor FISH study comparing breast ductal carcinoma in situ (DCIS) to synchronous invasive ductal carcinomas (IDCs). We observed 62.3 patterns (range, 14.5 to 93.3) in the DCIS and 70.6 patterns (range, 49.7 to 98.0) in the IDC.

In the breast lesions, we never observed any signal pattern clone comprising $>22\%$ of the tumor cell population, with 7 of 26 cases showing no stable signal pattern clone (ie, $<4\%$ of the cells have the same signal pattern), whereas in the prostate carcinomas, 4 of 13 cases showed signal pattern clones comprising $>22\%$ of the cells, with a range from 7% to 44% cells (average size, 17.5%) for the major signal pattern clones in all cases.

Although the higher instability indices and smaller clone populations observed in the breast tumors could be due to the fact that a different set of genes was analyzed, there are indications of generally higher intratumor heterogeneity in breast tumors, especially because certain breast tumors showed major clones with aberrations in all eight breast cancer–specific genes assessed. None of the prostate cancers showed major clones with more than three aberrations in the six genes assessed for prostate cancer progression, which might be indicative of lower intratumor heterogeneity in prostate tumors. We are currently analyzing cervical cancers and high-grade cervical intraepithelial neoplasias with eight gene probes specific for cervical cancer and have observed much lower instability indices in these tumors compared with the breast tumors and prostate tumors (data not shown). Taken together, these observations might indicate that there are major differences in tumor heterogeneity and clonal development between different tumor entities.

Although the instability index values between the prostate carcinomas with or without progression are similar, the frequency of chromosomal gains and losses is substantially higher in the progresser group, with 1.9 gains or losses per case versus 0.5 gains or losses in the non-progressers. By using the same threshold of $>30\%$ of the tumor cell population exhibiting the aberration, breast tumors showed much higher gain and loss frequencies, with an average of 3.5 gains and losses per DCIS case and 4.6 gains and losses per IDC case. The increase of aberrations in the more
advanced disease stages shows that, in both tumor entities, additional aberrations are acquired during progression of the disease.

Of the 13 prostate cancers investigated, 12 had major clonal cell populations with a TMPRSS2-ERG fusion and there was no significant difference in the average number of cells with a TMPRSS2-ERG fusion between prostate carcinoma cases with or without progression after RP. TMPRSS2-ERG fusion status could, therefore, not be used to discern progressors from non-progressors. This observation is in concordance with previous publications,\textsuperscript{16,18} and our results need to be interpreted with caution for ERG-negative cases. One progresser lesion (case 7) did not have any fusion event, but instead overexpressed ERG by acquiring extra copies of a normal ERG allele. This case was also the only case with CEP8 and MYC gains, indicating that this cancer followed a different pathway. Interestingly, Toubaji et al\textsuperscript{67} observed that increased gene copy number of ERG, but not TMPRSS2-ERG fusion, predicts outcome in prostate cancers. They found that the presence of extra copies of the ERG gene is significantly associated with recurrence, which is consistent with the observation that our only case with ERG gain (case 7) was actually a lesion from a patient who progressed.

Two non-progressers, but none of the progressors, revealed clonal cell populations with a double deletion of the sequences between ERG and TMPRSS2 (cases 4 and 6) (Figure 1), an ERG pattern that was reported to correlate with worse outcome.\textsuperscript{15} In our cases, the double-deletion pattern 202 happened in tetraploid cells, so the dosage effect in these cells is most likely similar to pattern 101 in diploid cells.

The most frequent aberration that we observed in prostate carcinomas that progressed was the loss of PTEN (Figure 3). Four of seven progressors had cell populations with >30% showing this loss, whereas none of the nonrecurrent cases reached this threshold. Loss of PTEN has been frequently shown to be associated with tumor progression, tumor aggressiveness, and disease recurrence,\textsuperscript{68,69} and appears to be a promising marker to distinguish between progressing and non-progressing prostate carcinomas.\textsuperscript{32,70} Interestingly, Leinonen et al\textsuperscript{18} observed that the loss of PTEN expression was associated with shorter progression-free survival in ERG-positive, but not in ERG-negative, cases. This is consistent with our findings, because all our cases with PTEN loss were ERG positive and progressors. Other gene markers that showed differential gain and loss patterns between progressors and non-progressors were TBL1XR1 on 3q26, CTNNBP2 on 7q31, and MYC on 8q24; however, this occurred to a much lesser degree than PTEN (Figure 3). Two of the markers, MEN1 on 11q13 and PDGFB on 22q13, did not show any differential gain and loss between progressors and non-progressors in our FISH analysis. MEN1 was gained in one non-progresser and one progressor, whereas none of the lesions showed PDGFB aberrations in >30% of the tumor cell population (Figure 3).

Herein, a probe set consisting of PTEN, MYC, and TBL1XR1 detected six of the seven progressors (86% sensitivity). The only progresser case that would not have been detected had a major clone with a TMPRSS2-ERG fusion pattern of 111 only, so additional markers might be needed to identify similar cases. However, none of these three genomic markers would have been positive for non-progressors, which is equivalent to a test specificity of 100%. Therefore, the combination of these three markers shows potential to predict progression in prostate carcinomas with high specificity and sensitivity. We successfully hybridized this probe panel on tissue sections of prostate cancers and detected, in one of the cancer specimens tested, a biallelic deletion of PTEN (ie, no signal for PTEN) and possible gains of the other two markers. This demonstration of the probe panel on prostate cancer tissue sections indicates that the proposed test is feasible and can be useful in a routine pathological setting. We plan to evaluate this probe panel in a larger study to further explore the prospect for a single-cell FISH test for the identification of patients with prostate cancer predicted to have a poor prognosis after RP and, thus, candidates for adjuvant therapy.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.06.030.

References


