A Three-Marker FISH Panel Detects More Genetic Aberrations of AR, PTEN and TMPRSS2/ERG in Castration-Resistant or Metastatic Prostate Cancers than in Primary Prostate Tumors

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Abstract

TMPRSS2/ERG rearrangement, PTEN gene deletion, and androgen receptor (AR) gene amplification have been observed in various stages of human prostate cancer. We hypothesized that using these markers as a combined panel would allow better differentiation between low-risk and high-risk prostate cancer. We analyzed 110 primary prostate cancer samples, 70 metastatic tumor samples from 11 patients, and 27 xenograft tissues derived from 22 advanced prostate cancer patients using fluorescence in situ hybridization (FISH) analysis with probes targeting the TMPRSS2/ERG, PTEN, and AR gene loci. Heterogeneity of the aberrations detected was evaluated. Genetic patterns were also correlated with transcript levels. Among samples with complete data available, the three-marker FISH panel detected chromosomal abnormalities in 53% of primary prostate cancers and 87% of metastatic (Met) or castration-resistant (CRPC) tumors. The number of markers with abnormal FISH result had a different distribution between the two groups (P<0.001). At the patient level, Met/CRPC tumors are 4.5 times more likely to show abnormalities than primary cancer patients (P<0.05). Heterogeneity among Met/CRPC tumors is mostly inter-patient. Intra-patient heterogeneity is primarily due to differences between the primary prostate tumor and the metastases while multiple metastatic sites show consistent abnormalities. Intra-tumor variability is most prominent with the AR copy number in primary tumors. AR copy number correlated well with the AR mRNA expression (rho = 0.52, P<0.001). Especially among TMPRSS2/ERG fusion-positive CRPC tumors, AR mRNA and ERG mRNA levels are strongly correlated (rho = 0.64, P<0.001). Overall, the three-marker FISH panel may represent a useful tool for risk stratification of prostate cancer patients.

Introduction

The discovery of recurrent ETS gene rearrangements in prostate cancers has led to studies evaluating the functional role of ETS genes in the pathogenesis of this disease and as diagnostic and prognostic biomarkers. The most common type of ETS rearrangement, the fusion of androgen-regulated TMPRSS2 with the oncogenic ERG is detected in approximately half of prostate tumors but none of benign glands [1]. However, studies assessing the prognostic significance of TMPRSS2:ERG fusion have yielded inconsistent results [2–5]. Additional genetic factors are likely to work in concert with the fusion during cancer progression. Recent studies have shown that genetic aberrations are not only common in prostate cancer but also interact with each other through related pathways, thereby contributing to the progression to invasive diseases. TMPRSS2 is regulated by androgens, and the androgen receptor (AR) is often amplified in patients treated with androgen deprivation therapy [6,7]. PTEN deletion, another common aberration in prostate cancer, was correlated with the expression of downstream p-Akt and associated with cancer-specific mortality [8,9]. ETS gene rearrangements were shown to cooperate with PTEN deletion and impact prostate cancer prognosis [10,11]. Crosstalk between PI3K and AR signaling pathways was recently suggested as a mechanism for the development of castration resistant prostate cancer (CRPC) [12,13]. PTEN deletion was shown to suppress androgen-responsive gene expression by modulating AR transcription factor activity. Also, PTEN and AR expression has been shown to inversely correlate in prostate cancer [14].
A critical clinical question concerns identifying characteristics of newly diagnosed prostate cancers that will distinguish aggressive from indolent behavior. The molecular heterogeneity of prostate cancers suggests that individual biomarkers may not be sufficient, and that multiple genetic markers may better associate with outcome. In the present study, we used a three-marker fluorescence in situ hybridization (FISH) panel to detect TMPRSS2 and/or ERG rearrangements, AR gene amplification, and PTEN deletion in both primary and CRPC prostate cancer samples and compared the prevalence, concurrence, and interaction of these three markers. With the reference of mRNA expression data generated from matching tumor samples from the same patient, we also demonstrated how FISH findings correlated with changes in gene expression. Intra- and inter-patient tumor heterogeneity was also analyzed.

Materials and Methods

Sample Acquisition

Ethics Statement. The study was approved by the Institutional Review Boards (IRB) of the Fred Hutchinson Cancer Research Center and the University of Washington Medical Center. IRB waived the need for written consent for this study because only de-identified materials were used, which were from the University of Washington Urology tissue bank.

Patient samples. De-identified archived untreated primary prostate cancer samples (n = 110) were obtained from the University of Washington (UW) and Virginia Mason Hospital in Seattle. A total of 83 primary tumors generated analyzable data for at least one FISH marker in the panel, including 69 patients with TMPRSS2/ERG FISH data, 65 patients with AR FISH data and 42 patients with PTEN FISH data. Metastatic tumor samples (n = 70) were collected at UW from autopsies performed within 2 to 4 hours of death of 11 CRPC patients under the rapid autopsy program [15]. Tumors were obtained from various organ sites, frozen immediately and stored at −80°C. All tissues were sectioned for H&E staining and, for verification of histology, reviewed by a pathologist. FISH analysis was focused on cancer areas. A total of 67 tumors yielded analyzable data for at least one FISH marker in the panel, including 56 tumors from 10 patients with TMPRSS2/ERG FISH data, 63 tumors from 11 patients with AR FISH data, and 62 tumors from 11 patients with PTEN FISH data.

Prostate cancer xenografts. Prostate cancer xenografts (LuCaP lines) were originally isolated from various organs of advanced patients [16]. FISH analyses were successful on 27 LuCaP lines, representing 22 patients, one of which was also among the metastatic patients described above. These included 27 tumors from 22 patients with TMPRSS2/ERG FISH data, 26 tumors from 21 patients with AR FISH data, and 25 tumors from 21 patients with PTEN FISH data. Together, combining metastatic patient tumors and xenografts derived from advanced-stage prostate cancer patients, the current study evaluated a total of 94 tumors from 32 patients.

Fluorescent In Situ Hybridization (FISH)

TMPRSS2/ERG rearrangement was assessed using our novel 4-color FISH assay as described separately [17]. “TMPRSS2/ERG” refers to the presence of fusion of the two genes. “TMPRSS2/ERG rearrangement” refers to various subtypes of rearrangement of either or both genes as specified in the Results section. FISH analysis of AR gene amplification was performed using the SpectrumOrange AR (Xq12) probe combined with the SpectrumGreen labeled Chr10 centromere (Xp11.1–1q11.1) CEP X probe as the control (Abbott Molecular, IL). PTEN gene deletion was examined using the PTEN/CEP10 dual-color FISH Probe set (Abbott Molecular, IL), including the SpectrumOrange labeled PTEN (10q23) probe and the SpectrumGreen labeled Chr10 centromere (10p11.1–1q11.1) CEP 10 probe.

For each sample, a range of 25 to 30 intact and non-overlapping interphase nuclei were enumerated manually using a 100× oil immersion lens on a Zeiss Z1 microscope (Carl Zeiss Canada Ltd, Canada). AR gain and PTEN deletion were assessed by counting the number of gene signal and the corresponding centromere signal per nucleus. AR gain was defined as an average copy number of AR per nuclei equal or higher than 2. True AR gene amplification was defined as the ratio of the total number of AR signals divided by the total number of the X-chromosome centromere equal or greater than 2. Samples with PTEN heterozygous deletion had a ratio of the total number of PTEN signals divided by the total number of CEP10 signals equal or below 0.75. A PTEN/CEP10 ratio equal or below 0.2 is considered homozygous PTEN deletion. For patient-level analyses of CRPC patients with multiple tumors, expression by a given marker was considered abnormal if the aberration was seen in at least one tumor.

Expression Array

Agilent 44 K whole human genome expression oligonucleotide microarrays (Agilent Technologies, Inc., Santa Clara, CA) were used to profile prostate cancer xenografts and human castration-resistant soft tissue metastases of prostate. Freshly frozen xenografts were processed to extract total RNA which was amplified one round; patient samples were laser-capture micro-dissected and amplified two rounds as described previously [18]. Probe labeling and hybridization was performed following the Agilent suggested protocols and fluorescent array images were collected using the Agilent DNA microarray scanner G2565BA. Agilent Feature Extraction software was used to grid, extract, and normalize data. Expression ratios were log, scaled and mean-centered across each gene.

Statistical Analysis

To complement the comparisons of archived primary tumor with a separate cohort of patients with metastatic disease, we examined within-patient heterogeneity of AR and PTEN for patients with metastatic disease, hypothesizing that prostate tumors could differ from contemporaneous metastatic lesions. Linear mixed models with random patient effects were fitted to non-prostate tumors, and a 95% confidence interval calculated for subject-specific [19] predictions of average expression. If a subject’s prostate tumor copy number status fell outside the confidence interval, it would be interpreted as evidence of potential differences between the copy number status of primary and metastatic lesions. A linear mixed models effect model and the %ICC9 SAS macro was used to calculate intraclass correlation coefficients and their confidence intervals [20]. Logistic regression and generalized estimating equations (GEE) were used to compare rates of abnormality for primary and metastatic samples, controlling for tissue source (rapid autopsy vs xenograft) and within-patient correlation for tumor-level analysis. Heterogeneity of intratumoral variance for different tumor sites was also explored using linear mixed models. Additional statistical inference included Spearman correlation coefficients, and the Wilcoxon rank sum test to compare distributions of the number of markers with abnormal expression. P-values were two-sided; statistical analyses were conducted using SAS/STAT software, version 9.3 (SAS Institute, Inc., Cary, NC).
Results

The Prevalence of Genetic Aberrations Detected by the Three-marker FISH Panel in Localized Primary and Metastatic or Castration Resistant (Met/CRPC) Patients

The three-marker FISH panel (Figure 1) used in our study detected frequent genetic aberrations in prostate cancer, and these were significantly more common in Met/CRPC tumors than in untreated primary tumors (Figure 2A).

Of the 34 primary tumors in which all 3 markers could be assessed, 16 (47%) exhibited no aberrations involving AR, PTEN, or TMPRSS2/ERG, 11 (32%) were abnormal by one marker only. Six patients’ tumors (18%) were detected abnormal by two markers, including 3 with TMPRSS2/ERG fusion and homozygous PTEN deletion, 2 with TMPRSS2/ERG fusion and heterozygous PTEN deletion, and 1 with non-fusion alternative rearrangement along with heterozygous PTEN deletion. None of the patients were abnormal by all three markers because there was no detectable AR abnormality when the cutoff for AR gain was set to > = 2.0 AR per nucleus, an arbitrarily determined stringent cutoff. Two patients would be classified as mild AR gain if using AR copy number per nucleus > = 1.5 as the cutoff value, established as mean+3SD based on enumeration results on normal prostate epithelial cells from 18 different samples.

Of the 30 Met/CRPC patients/xenografts with FISH results from all three markers, 18 (60%) included 5 (17%) that resulted from gain of PTEN (75%) also showed an abnormal TMPRSS2/ERG deletion (93%) also showed abnormalities in TMPRSS2/ERG FISH analysis. In Met/CRPC patients, abnormal TMPRSS2/ERG FISH results were also more prevalent among patients demonstrating gain of AR, and vice versa (Figure 2E). Among the 30 Met/CRPC patients with data available from with either both or all three markers, 13 of the 14 individuals with PTEN deletion (93%) also showed abnormalities in TMPRSS2/ERG FISH abnormalities. Sixteen of 24 patients (67%) with TMPRSS2/ERG abnormalities also demonstrated AR gain. Detailed FISH results on all metastatic samples from each CRPC patient are summarized in Table 2. Results of xenograft samples are listed in Table 3.

Intra- and Inter-patient Comparison of Genomic Aberrations and Heterogeneity in Castration Resistant Prostate Cancer

The 3-marker FISH analyses yielded two observations from patients with metastatic prostate cancer: (1) within the same patient, aberrations in metastatic tumors were generally consistent across tumors; (2) several primary prostate tumors of CRPC patients exhibited a profile distinct from distant metastatic sites.

Nine patients (27%) were detected as abnormal by two markers, including 8 (27%) shown as abnormal by TMPRSS2/ERG and AR FISH and 5 (17%) by TMPRSS2/ERG and PTEN. Eight patients (27%) were abnormal by all three tests.

We further evaluated subtypes of genetic aberrations detected by each marker in the Met/CRPC cohort (Figure 2 B-D). Rearrangements of TMPRSS2 and/or ERG were detected in 14 patients (47%), including 5 (17%) with the typical single TMPRSS2/ERG fusion, 5 (17%) with dual or complex TMPRSS2/ERG fusion, and 4 (13%) with alternative rearrangements without fusion. Copy number increase (CNI) of chromosome 21 was observed in 10 patients (33%) using the TMPRSS2/ERG FISH probes. AR gain in one or more lesions was observed in 18 patients (60%), including 6 (20%) that resulted from gain of the X-chromosome and 12 (40%) with true AR gene amplification (AR/X > = 2). Deletion of PTEN was detected in 15 patients (50%), including 5 with homozygous deletion.

A Wilcoxon rank sum test suggested that the Met/CRPC cohort (n = 30) generally had more alterations detected by FISH than the cohort of primary cancers (N = 34) (W = 1287, P<0.001). AR gain, including moderate gain (W = 1334, P<0.001), and the combination of TMPRSS2/ERG and PTEN alterations (W = 1181, P = 0.005) were also significantly more common in the Met/CRPC tumors.

The investigation of individual markers reflected unique trends of changes of each genetic abnormality during the progression of prostate cancer. About 80% of Met/CRPC samples were identified by TMPRSS2/ERG FISH as abnormal, compared to 48% in primary samples, and the difference was statistically significant (Table 1; Figure 2B) (P = 0.03). This difference appeared to be due to the CNI aberration rather than the TMPRSS2/ERG fusion itself; the percentage of patients with fusion or alternative rearrangement remains similar, but the percentage of patients with dual fusion as opposed to single fusion is clearly greater in the Met/CRPC category than in the primary tumor group (Figure 2B). Examining individual tumors (adjusting for with-person correlation and xenograft status), the odds of a Met/CRPC tumor exhibiting an abnormality was 4.5 times greater than odds for a primary tumor (P = 0.05). While nearly all primary cancer patients showed normal AR status, over 70% of Met/CRPC patients demonstrated various degrees of AR gene copy number gain (Table 1; Figure 2C). PTEN FISH showed increased heterozygous PTEN deletion and homozygous PTEN deletion in Met/CRPC compared with primary patients (Table 1; Figure 2D) (P = 0.07 at the tumor level, P = 0.005 at the patient level). Of note, for patient-level assessments there was a hierarchy, so if one lesion was heterozygous and the other homozygous, the patient level was considered homozygous.

Data of the entire panel across different individuals showed that prostate cancer patients with PTEN deletion also tended to exhibit abnormal results in TMPRSS2/ERG FISH (Figure 2E). Among the 34 primary cancer patients with data available from all three markers, 6 of the 8 individuals with PTEN deletion (75%) also showed an abnormal TMPRSS2/ERG FISH result (Figure 2E). Among the 30 Met/CRPC patients with data available from with either both or all three markers, 13 of the 14 individuals with PTEN deletion (93%) also showed abnormalities in TMPRSS2/ERG FISH analysis. Sixteen of 24 patients (67%) with TMPRSS2/ERG abnormalities also demonstrated AR gain. Detailed FISH results on all metastatic samples from each CRPC patient are summarized in Table 2. Results of xenograft samples are listed in Table 3.

In other cases (#8 and #10), prostate tumors did not differ from metastatic lesions in abnormal vs normal marker signals, but were outside of the 95% confidence interval for the subject-specific average based on linear mixed models fit to metastatic lesions.

In general, more than 75% of the variability was between-patient, with relatively little within-patient variation: the intraclass correlation coefficient was 0.76 (95% CI 0.54–0.90) for average AR and 0.82 (95% CI 0.62–0.92) for PTEN/CEP10. However, when the entire panel was evaluated for each individual, 4 (#3, #5, #9 and #11) out of 8 patients (50%) with data available from all three markers in the local prostate tumors showed different profiles between the primary and metastatic tumors. Further comparison using data of individual markers showed different levels of deviation of the primary from metastatic tumors. Of the 8 patients with available TMPRSS2/ERG FISH results on prostate site
tumors, 3 (37.5%) had results in the prostate different from those in other metastatic sites (Table 2). Interestingly, patient #5 demonstrated dual deletion fusion among metastatic sites, while only single deletion fusion was detected in a tumor from the prostate of the same patient. In the analyses of AR, 3 (#3, #9, and #11) out of 10 patients (30%) showed results in the prostate that...
Figure 2. The prevalence of genetic aberrations detected by the panel. A patient with multiple tumors was considered abnormal by a given marker if the aberration was seen in at least one tumor. (A) Pie charts demonstrate the percentage of individuals with no (white), one (light grey), two (dark grey), and three (black) abnormalities detected by the panel among the primary prostate cancer (n = 34) and the metastatic or castration resistant prostate cancer (Met/CRPC) cohort (n = 30), respectively. Among primary patients, an asterisk was used to highlight moderate AR gain (average AR per nucleus $\geq 1.5$ but $< 2$). (B-D) Prevalence of each subtype of abnormalities detected by individual FISH marker among the primary patients (one tumor per patient, n = 34), Met/CRPC tumors (n = 81), and Met/CRPC patients/xenografts (n = 30), respectively. TMPRSS2/ERG abnormalities are categorized as single fusion (light blue), dual/complex fusion (dark blue), alternative rearrangements (green), and copy number increase (CNI) of the normal gene alleles (yellow). AR FISH detected moderate AR gain (light blue), gain of X (dark blue) and AR gene amplification (green). PTEN FISH abnormalities includes heterozygous (light blue) and homozygous (green) PTEN deletions. (E) The co-occurrence of abnormalities in the three markers shown as 3D sphere plots for the primary cancer cohort (left) and the Met/CRPC cohort (right). TMPRSS2/ERG, PTEN, and AR results are presented on X, Y, and Z axes, respectively. The value presented on each axis ranges from 0 to 1. “0” denotes normal for a given marker. For TMPRSS2/ERG, “0.5” indicates rearrangements, including fusion and alternative rearrangements; “1” means CNI of the normal alleles without any rearrangement. For PTEN FISH, both heterozygous and homozygous deletions are presented as “1”. For AR FISH, “1” indicates AR copy number gain ($\geq 2.0$). Patients with the same combination of abnormalities are clustered into a sphere, the volume of which is proportional to the percentage of patients in the respective cohort. Only patients with available data from all three markers are included. The green sphere in the primary patient plot denotes moderate AR gain ($1.5$ but $< 2.0$).
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deviated from extra-prostatic tumors. The assessment of PTEN deletion showed that 2 (#3 and #11) out of 9 patients (22.2%) demonstrated different PTEN FISH results between prostate site and metastatic tumors. In patient #11, all extra-prostatic metastasis showed homozygous PTEN deletion, while prostate tumors showed normal PTEN results. Similarly, the panel data for xenografts also indicated that xenograft lines derived from the same patient tend to show the same genomic abnormality (Table 3).

Intra-tumoral Assessments of Genomic Heterogeneity in Metastatic Patients

We next sought to evaluate variation in genomic alterations detected by the FISH panel in individual cells comprising a primary or metastatic prostate cancer (Met/CRPC) patients. The assessment of PTEN deletion showed that 2 (#3 and #11) out of 9 patients (22.2%) demonstrated different PTEN FISH results between prostate site and metastatic tumors. In patient #11, all extra-prostatic metastasis showed homozygous PTEN deletion, while prostate tumors showed normal PTEN results. Similarly, the panel data for xenografts also indicated that xenograft lines derived from the same patient tend to show the same genomic abnormality (Table 3).

Table 1. Prevalence of abnormalities detected by each FISH marker among the primary and the metastatic or castration-resistant prostate cancer (Met/CRPC) patients.

|                      | Primary # of patients | # of tumors | Met/CRPC # of patients | Met/CRPC Tumors | Patients |
|----------------------|-----------------------|-------------|------------------------|-----------------|----------|
|                       | %                     | %           | %                      |                 |          |
| **TMPRSS2/ERG**       |                       |             |                        |                 |          |
| Single fusion         | 69                    | 82          | 31                     | 0.05            | 0.03     |
| Dual/complex fusion   | 25                    | 36%         | 13                     | 16%             | 5        |
| Alternative rearrangement without fusion | 2                   | 3%          | 25                     | 30%             | 5        |
| Copy number increase  | 4                     | 6%          | 9                      | 11%             | 4        |
| Normal                | 36                    | 52%         | 17                     | 21%             | 7        |
| **AR**                |                       |             |                        |                 |          |
| Moderate AR gain      | 2                     | 3%          | 17                     | 19%             | 6        |
| Gain of X             | 63                    | 97%         | 23                     | 26%             | 7        |
| AR amplification      | 42                    | 14%         | 20                     | 23%             | 9        |
| Normal                | 3                     | 7%          | 16                     | 19%             | 5        |
| **PTEN**              |                       |             |                        |                 |          |
| Heterozygous         | 6                     | 14%         | 20                     | 23%             | 9        |
| HOMOZYGOUS PTEN deletion | 3               | 7%          | 16                     | 19%             | 5        |
| Normal                | 33                    | 79%         | 50                     | 58%             | 17       |

1. Average AR per nucleus ≥1.5 but <2.
2. Average AR per nucleus ≥2 but average AR/X ratio <2.
3. Average AR/X ratio ≥2.
4. Average PTEN/CEP10 ratio ≤0.75 but >0.2.
5. Average PTEN/CEP10 ratio ≥0.2.
6. Wald tests of abnormal vs. normal for primary vs. CRPC, generalized estimating equations (GEE) with independence autocorrelation, adjusting for rapid autopsy vs xenograft sample for CRPC. Likelihood ratio test for AR, since no primary samples had abnormal AR.
7. Wald tests of abnormal vs. normal for primary vs. CRPC, logistic regression adjusting for rapid autopsy vs xenograft sample for CRPC. Likelihood ratio test for AR, since no primary samples had normal AR.

Table 4 suggests that the tissue-based heterogeneity differences were due to low within-patient variation in the peritoneal and adrenal lesions. These effects may be confounded with patient effects since few patients had adrenal or peritoneal lesions. By a likelihood ratio test, statistical models with separate covariate estimates for each tumor type fit the data better than a model that did not distinguish between tumors (χ² = 412, P<0.001), and a model that distinguished between prostate tissue and other lesions (χ² = 332, P<0.001).

Correlation of Genomic Alterations and Gene Expression in Castration Resistant Prostate Cancer

In order to investigate the functional relationship of genetic aberrations detected by our panel, we correlated our FISH findings with gene expression data from 91 matching Met/CRPC samples, including 65 patient tumors and 26 xenografts (Table S1). We first compared AR copy number, determined by FISH, with the AR transcript abundance, determined by cDNA microarray, from the same tumor sample. We observed a wide range of AR expression in Met/CRPC tumors (Figure 4A). The average number of AR per nucleus and the level of relative AR mRNA were positively correlated with rho = 0.52 (P<0.001) (Figure 4A). When normalized to the median AR mRNA expression level of all tumors with both AR FISH and mRNA expression data (n = 88),
samples with \( AR \) gain (\( n = 48 \)), including gain of \( X \) (\( n = 17 \)) and \( AR \) amplification (\( n = 31 \)) expressed \( AR \) mRNA at \( 2.5 \pm 0.3\)-fold (Mean\( \pm \)S.E.) higher than the median, while tumors without \( AR \) gain (\( n = 40 \)) had \( AR \) mRNA level as \( 0.7 \pm 0.2\)-fold comparing to the median (\( W = 1106, P < 0.001 \)). When tumors with \( AR \) gain were further divided into groups of gain of \( X \) (\( n = 17 \)) vs \( AR \) gene amplification (\( n = 31 \)), our data showed that \( AR \) mRNA was expressed at a similar level between the two (\( W = 361, P = 0.24 \)).

We then assessed the effect of \( TMPRSS2:ERG \) fusion on \( ERG \) mRNA levels and evaluated whether \( ERG \) expression also associated with the \( AR \) abundance in Met/CRPC tumors (\( n = 80 \), Figure 4B). Fusion-negative tumors (\( n = 42 \)) expressed

### Table 2. FISH data of individual castration resistant metastatic patient tumors.

| Patient Tissue | TMPRSS2/ERG | Average AR per nucleus | AR/X | PTEN/CEP10 |
|----------------|-------------|------------------------|------|------------|
| 8 Liver        | Normal      | 5.50                   | 3.27 | 1.19       |
| 8 LN1          | Normal      | 1.12                   | 1.00 | 1.04       |
| 8 LN2          | Normal      | 4.94                   | 2.74 | 1.20       |
| 8 LN3          | Normal      | 5.70                   | 3.35 | 1.16       |
| 8 Lung         | Normal      | 4.38                   | 2.74 | 1.12       |
| 8 Prostate     | Normal      | 2.52                   | 1.42 | 1.00       |
| 1 Liver        | Normal      | NA                     | NA   | 0.50       |
| 1 LN1          | Normal      | 1.00                   | 1.00 | 0.52       |
| 1 LN2          | Normal      | 1.03                   | 1.00 | 0.49       |
| 1 Prostate     | Copy number increase | 1.00 | 1.00 | 0.50       |
| 4 Liver        | Single fusion | 1.19                   | 1.00 | 0.00       |
| 4 LN1          | Single fusion | 1.05                   | 0.98 | 0.00       |
| 4 Lung1        | Single fusion | 1.00                   | 1.00 | 0.00       |
| 4 Lung2        | NA          | 1.11                   | 1.00 | NA         |
| 4 Spleen       | Single fusion | 1.04                   | 1.00 | 0.00       |
| 4 Prostate     | NA          | 1.08                   | 1.00 | NA         |
| 5 LN1          | Dual/complex fusion | 20.37                  | 7.10 | 0.04       |
| 5 LN2          | Dual/complex fusion | 20.76                  | 6.18 | 0.05       |
| 5 LN3          | Dual/complex fusion | 37.48                  | 10.18 | 0.00   |
| 5 LN4          | Dual/complex fusion | 18.16                  | 6.78 | 0.08       |
| 5 LN5          | Dual/complex fusion | 14.48                  | 6.58 | 0.10       |
| 5 Prostate     | Single fusion | 102.64                  | 54.60 | 0.03    |
| 2 LN1          | Dual/complex fusion | 1.10                   | 1.00 | 1.00       |
| 2 LN2          | Dual/complex fusion | 1.66                   | 0.99 | 0.80       |
| 2 LN3          | Dual/complex fusion | 1.92                   | 1.02 | 0.97       |
| 2 Lung1        | Dual/complex fusion | 1.22                   | 1.00 | 0.96       |
| 2 Lung2        | Dual/complex fusion | 2.00                   | 1.00 | 0.98       |
| 2 Prostate     | Dual/complex fusion | 1.75                   | 1.00 | 0.95       |
| 9 Adrenal1     | Dual/complex fusion | 1.62                   | 1.09 | 1.02       |
| 9 Adrenal2     | Dual/complex fusion | 1.62                   | 1.09 | 1.02       |
| 9 Liver        | Dual/complex fusion | 1.50                   | 0.99 | 0.96       |
| 9 LN1          | Dual/complex fusion | 1.86                   | 1.06 | 0.96       |
| 9 LN2          | Dual/complex fusion | 1.50                   | 0.96 | 1.00       |
| 9 LN3          | Dual/complex fusion | 1.28                   | 0.98 | 1.02       |
| 9 LN4          | Dual/complex fusion | 1.26                   | 1.02 | 0.90       |
| 9 Lung1        | Dual/complex fusion | NA                     | NA   | 0.94       |
| 9 Lung2        | Dual/complex fusion | 1.48                   | 1.04 | 1.04       |
| 9 Spleen       | Dual/complex fusion | 1.64                   | 1.01 | 0.86       |
| 9 Prostate     | Dual/complex fusion | 14.22                  | 6.35 | 0.95       |
| 7 LN1          | Alternative rearrangement | 8.20                   | 3.20 | 0.73       |
| 7 LN2          | Alternative rearrangement | 16.48                  | 9.81 | 0.80       |
| 7 LN3          | Alternative rearrangement | 17.32                  | 8.33 | 0.82       |
| 7 LN4          | Alternative rearrangement | 37.64                  | 12.38 | 0.50   |

Only samples successfully hybridized with at least one marker were presented in the table, including 56 tumors with \( TMPRSS2/ERG \) FISH, 65 tumors with \( AR \) FISH, and 62 tumors with \( PTEN \) FISH results.

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ERG mRNA at 0.7±0.1 relative to the probe median, while fusion-positive tumors (n = 38) expressed significantly higher ERG mRNA at 910.8±3.2 fold relative to probe median (W = 2073, P<0.001). Copy number increase (CNI) of ERG (or of both TMPRSS2 and ERG without fusion) did not associate with higher ERG mRNA expression.

As ERG expression in the context of a TMPRSS2:ERG fusion is regulated by AR activity, we evaluated the effect of AR on ERG expression in Met/CRPC tumors with and without TMPRSS2:ERG fusion. While both the fusion-positive and fusion-negative samples showed a significant correlation between AR mRNA and ERG mRNA expression, this correlation appeared stronger in fusion-positive samples (rho = 0.64, P<0.001, n = 38) than in fusion-negative samples (rho = 0.36, P = 0.02, n = 42). This correlation was further confirmed by a dichotomized comparison of ERG expression levels for the 38 fusion-positive samples between low- and high-AR mRNA expression groups using the median probe intensity as a divider. The low AR expressing tumors (n = 20) expressed ERG at 3.6±1.1 fold relative to the probe median, while the high AR expressing tumors (n = 18) expressed ERG at 21.2±5.8 fold of probe median (P<0.01).

**Discussion**

A Three Marker FISH Panel Detects High Rates of Recurrent Genomic Aberrations in Localized and Metastatic Prostate Cancers

Because of the controversial prognostic utility of TMPRSS2:ERG fusion in prostate cancer, we employed the strategy of a three-marker FISH panel to detect well documented prostate cancer DNA aberrations, including TMPRSS2/ERG rearrangements, AR copy number gain, and PTEN deletion. This panel clearly detected a significant number of genetic abnormalities in prostate carcinomas, 53% in primary tumors and 87% in Met/CRPCs. At the individual tumor level, the odds of a Met/CRPC tumor being abnormal were 4.5 times greater than that for a primary tumor. Collectively, if aberrations in these genomic loci associate with aggressive tumor behavior, then this three-marker FISH panel may be a useful tool in distinguishing high-risk patients from low-risk ones at diagnosis or in repeat assessments using active surveillance strategies. In addition, this approach may be particularly useful in characterization of circulating and disseminated tumor cells (CTC/DTC) as using fewer cells for analysis.

### Table 3. FISH data of individual xenograft tumors.

| Xenografts | Tissue | TMPRSS2/ERG | Average AR per nucleus | AR/X | PTEN/CEP10 |
|------------|--------|-------------|------------------------|------|-----------|
| LuCaP81 | LN | Normal | 1.00 | 1.00 | 0.91 |
| LuCaP78 | Peritoneal | Normal | 1.04 | 1.00 | 1.00 |
| LuCaP136 | Acites fluid(cells) | Normal | 1.04 | 1.00 | 0.00 |
| LuCaP1531 | NA | Normal | 1.50 | 1.00 | 1.63 |
| LuCaP147 | Liver | Normal | 1.96 | 1.96 | 1.00 |
| LuCaP49 | Omental fat met | Single fusion | 1.10 | 0.97 | 0.54 |
| LuCaP86.2 | Bladder | Single fusion | 1.97 | 1.00 | 0.93 |
| LuCaP23.12 | Liver | Single fusion | 2.20 | 1.04 | 0.89 |
| LuCaP23.1CR | LuCaP23.1 | Single fusion | 2.28 | 1.00 | NA |
| LuCaP23.1 | LN | Single fusion | 2.48 | 1.00 | 0.95 |
| LuCaP35 | LN | Single fusion | 6.44 | 2.98 | 0.91 |
| LuCaP35CR | LuCaP35 | Single fusion | 34.76 | 12.78 | 0.96 |
| LuCaP145.1* | Liver | Single fusion | 1.60 | 1.00 | 1.15 |
| LuCaP145.2* | LN | Dual/complex fusion | 1.79 | 0.99 | 0.87 |
| LuCaP93 | Prostate | Dual/complex fusion | 1.50 | 0.99 | 0.00 |
| LuCaP92 | Peritoneal | Dual/complex fusion | 2.00 | 1.00 | 0.90 |
| LuCaP58 | LN | Alternative rearrangement | 1.52 | 1.00 | 0.37 |
| LuCaP96** | Prostate | Alternative rearrangement | 1.52 | 1.03 | 0.60 |
| LuCaP96CR | LuCaP96 | Alternative rearrangement | 5.72 | 3.33 | 0.79 |
| LuCaP73 | Prostate | Copy number increase | 1.48 | 1.00 | 1.03 |
| LuCaP115 | LN | Copy number increase | 1.72 | 1.08 | 1.02 |
| LuCaP70 | Liver | Copy number increase | 2.08 | 1.00 | 1.00 |
| LuCaP141 | Prostate | Copy number increase | 2.64 | 1.00 | 0.98 |
| LuCaP146 | NA | Copy number increase | 6.80 | 3.90 | 1.16 |
| LuCaP69† | NA | Copy number increase | 16.70 | 7.50 | 0.53 |
| LuCaP105 | Rib | Copy number increase | 119.16 | 70.93 | 0.61 |

*Xenograft discontinued.

*Xenograft derived from patient #9 in Table 2.

**Xenograft derived from a patient with localized prostate cancer.

Only samples successfully hybridized with at least one marker were presented in the table, including 23 with TMPRSS2/ERG FISH, 26 with AR FISH, and 25 xenografts with PTEN FISH results.

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Within-person heterogeneity of AR and PTEN/CEP10 for rapid autopsy patients

**Table 4. Summary of intratumoral heterogeneity.**

| Tumor Type       | N1   | Average AR per cell2 | Predicted AR per cell (95% confidence interval) | Predicted within-patient standard deviation | Average PTEN/CEP10 | Predicted PTEN/CEP10 (95% confidence interval) | Predicted within-patient standard deviation |
|------------------|------|----------------------|-------------------------------------------------|------------------------------------------|-------------------|-----------------------------------------------|------------------------------------------|
| Prostate         | 12   | 2.1                  | 3.0 (2.6–3.3)                                   | 1.43                                     | 0.9               | 0.8 (0.7–0.9)                                | 0.43                                     |
| Adrenal          | 2    | 1.6                  | 1.5 (1.4–1.6)                                   | 1.04                                     | 1.0               | 1.0 (0.9–1.1)                                | 0.21                                     |
| Liver            | 6    | 2.6                  | 2.3 (2.1–2.5)                                   | 1.12                                     | 0.7               | 0.7 (0.6–0.8)                                | 0.44                                     |
| Lymph Node       | 34   | 5.3                  | 3.5 (3.3–3.7)                                   | 1.29                                     | 0.5               | 0.7 (0.6–0.7)                                | 0.47                                     |
| Lung             | 10   | 2.0                  | 2.1 (2.0–2.3)                                   | 1.13                                     | 1.0               | 0.7 (0.6–0.8)                                | 0.46                                     |
| Peritoneal       | 1    | 2.2                  | 2.0 (1.7–2.3)                                   | 1.06                                     | 0.4               | 0.4 (0.2–0.6)                                | 0.29                                     |
| Spleen           | 2    | 1.3                  | 1.3 (1.2–1.3)                                   | 1.02                                     | 0.4               | 0.5 (0.3–0.7)                                | 0.54                                     |

1 Number of tumors in sample.
2 Median for analyzed tissue.

Predicted values and covariance parameter estimates are from linear mixed models predicting copy number by tumor type, with random patient effects and separate covariance parameter estimates (within-patient heterogeneity and measurement error) for each tumor type.

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**Figure 3. Within-patient heterogeneity of AR and PTEN/CEP10 for rapid autopsy patients (n = 11).** Each tumor’s FISH result is represented by a plotting character (grey for metastatic lesions, red for prostate) with multiple lesions in the same patient at the same X coordinate. Confidence intervals for subject-specific average copy number values are shown in black. Thresholds for abnormal signals are marked as horizontal dashed lines on each plot. (A) Average number of AR per nucleus. (B) Average PTEN/CEP10 ratio.

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and getting data on three specific markers would be a significant advantage. The utility of these three markers is further supported by findings from a recent study using whole exome and transcriptome sequencing technologies [21]. Grasso et al. identified that AR and PTEN had the highest level of copy number gains and losses, respectively, in prostate cancer, especially CRPC. Their integrated genomic approach also demonstrated the interplay of these genomic alterations with TMPRSS2/ERG rearrangements. For each individual marker, our study detected similar abnormality rates as reported in the literature. For rearrangements of TMPRSS2 and/or ERG, previous findings showed ERG rearrangements in 30–50% of localized prostate cancers [1,2,4,5,22] and 40–50% of metastatic diseases [4,23–25]. With our novel 4-color FISH technique, capable of detecting rearrangements of TMPRSS2 and/or ERG simultaneously in a single hybridization, we found the similar prevalence for TMPRSS2:ERG fusion, as well as non-fusion alternative rearrangements in 10–12% patients in both groups. However, dual/complex TMPRSS2:ERG fusion, which has been shown to associate with poor survival, occurs with a substantially greater frequency in Met/CRPC patients (17%) than in primary cancer patients (3%). Similarly, copy number increase (CNI) of TMPRSS2 and ERG without fusion was more frequent in Met/CRPC patients (33%) than in primary cancer patients (3%), suggesting increased genetic instability as the disease progresses, which was also observed in our studies of disseminated tumor cells obtained from prostate cancer patients [23,26].

To date, multiple studies have demonstrated the occurrence of PTEN loss ranging from less than 20% to nearly 70% in early stage prostate cancer [8,11,27,28]. The variation could be attributed to multiple factors such as differences in patient populations, cohort sizes, and the cutoffs used to determine the PTEN deletion. Setting the cutoffs (based on the percentage of abnormal nuclei among all nuclei scored) as 10% for homozygous and 40% for heterozygous deletion, Reid and colleagues identified 17% of untreated primary prostate cancers exhibiting heterozygous or homozygous deletion, Reid and colleagues identified 17% of untreated primary prostate cancers exhibiting heterozygous or homozygous deletion of PTEN [11]. Setting the cutoffs as 30% for homozygous and 20% for heterozygous deletion, Yoshimoto et al. identified the presence of heterozygous and homozygous PTEN deletion in 39% and 5% prostate cancer patients, respectively [8]. We observed PTEN deletion in 21% of primary cancer patients and 47% of the CRPC patients based on the average ratio of PTEN/CEP 10 signals. Similar to previous findings [29], our study found that PTEN deletion tumors also tended to harbor TMPRSS2/ERG abnormalities (Figure 2E).

Our findings on AR gene amplification are unique and particularly interesting. AR amplification is generally considered to be only associated with CRPC tumors, induced by hormonal deprivation therapy or treatment with AR antagonists. Previous FISH studies rarely detected AR gene amplification in clinically
localized prostate tumors before hormonal therapy, but gain of the X-chromosome has been reported in 30–50% patients when a cutoff for gain was set at 9.8% of all cells examined [30,31], which implied that an average of >1.1 copies of the X-chromosome per nucleus were considered abnormal. In recurrent prostate cancer, AR amplification was common, with the reported frequency varying between 20% and 60% [6,7,32,33]. In these studies, AR gene amplification was defined in a slightly different manner. For example, among the studies that used AR/X ratio to define the amplification, the cutoffs vary from 1.5 [32,33], 2.0 [6], to 3.0 [7]. In the present study, we separated the subtype of true AR gene amplification, defined as AR/X ratio >2.0, from general AR gain, defined as having an average AR per nucleus of >2.0. We found AR gain in 38% of Met/CRPC patients, including 39% presenting as true AR gene amplification and 19%, demonstrating AR gain due to simultaneous gain of the X-chromosome, with an average number of X-chromosomes per nuclei exceeding 2.0 (Table 1). There was, however, no difference in the AR mRNA expression between the groups of X-gain vs AR gene amplification; AR copy numbers correlated well with AR mRNA levels (Figure 3A). We also observed by SNP-array CGH analysis that the multiple X centromere signals observed by FISH sometimes represent only focal gain or amplification of the genomic region around the centromere of the X-chromosome including AR rather than gain of the entire X-chromosome (Schoenborn, unpublished data not shown). These data argue for using the absolute AR copy number alone to define AR gain/amplification in FISH studies regardless of the AR/X ratio. Consequently, using the cutoff of 2.0 for AR gain would mean that a tumor would be considered abnormal for AR gain when AR copy number is at least doubled (from one copy in normal male cells to two copies in cancer cells) in 100% of the cells, which might be too stringent a criterion and explains why AR gain was never reported previously in primary prostate cancer. Our experimental cutoff based on signal patterns seen in a series of normal controls was 1.48. Therefore, we used the 1.50 cutoff for moderate AR gain in Table 1, which translates to that AR gain in 50% of the cells would be considered abnormal. With this cutoff, we observed 6% of primary patients and 77% of Met/CRPC patients with AR gain. This should be a better definition for AR gain and may allow identification of primary prostate cancer patients with high risk for disease progression. Supporting evidence came from LaCaP 96 (Table 3), a xenograft line derived from a localized primary prostate cancer which showed moderate AR gain (1.52 AR per nucleus). Its castration-resistant derivative line LuCaP 96CR showed clear AR amplification (5.72 AR per nucleus). The original patient indeed had aggressive disease and died from prostate cancer. The caveat, however, is that the xenograft data may not faithfully represent the original tumor genomics due to potential selection pressures over time on the xenograft specimens.

**FISH Detected Genetic Abnormalities Strongly Correlate with Changes at the Expression Level and Suggest Functional Interactions between AR, PTEN and TMPRSS2/ERG**

Chaux et al. identified a strong association between ERG protein staining using immunohistochemistry and the TMPRSS2:ERG fusion status defined by FISH [34]. Similarly, our study showed that ERG mRNA expression was significantly correlated with the presence of TMPRSS2:ERG fusion. We also demonstrated a strong positive correlation between AR copy number gain and increased level of AR mRNA expression, supporting previous studies which showed higher levels of AR protein expression in prostate tumors with AR gene amplification than tumors without AR amplification [33]. Unlike this study that did not find an effect of X-chromosome gain on AR mRNA, we found higher AR mRNA levels in tumors with simple gain of X-chromosome, the amplitude of which could not be differentiated from tumors with true AR gene amplification. The similarity in AR mRNA levels in these two groups may in part be due to the nature of transcriptome array analyses, where the quantification of very high levels of AR mRNA reaches a plateau.

Related to the functional interactions of these genetic aberrations, previous studies demonstrated the cooperative relations between PTEN deletion and TMPRSS2/ERG rearrangements in animal models [29,35]. Clinical studies demonstrated significant correlations between PTEN gene deletion and deregulation of P-AKT as well as AR protein expression in advanced localized prostate cancer [9]. Two recent studies suggested cross-talk between androgen signaling pathway and the PI3K signaling in a reciprocal fashion [12,13]. At the genomic level, studies using large clinical cohorts demonstrated both presence and absence of enrichment between TMPRSS2:ERG fusion and PTEN gene deletion in prostate cancer [9,11]. Our study also confirmed enrichment of TMPRSS2:ERG abnormalities in tumors with either PTEN deletion or AR gain (Figure 2E). AR gain, but not PTEN deletion, was enriched in Met/CRPC tumors with TMPRSS2/ERG abnormalities. However, it is not obvious from our study that PTEN and AR expression were inversely correlated in prostate cancer, as previously reported [14].

More importantly, we demonstrated that AR and ERG expression levels strongly correlated with each other, especially in TMPRSS2:ERG fusion-positive tumors (Figure 4B). We propose the model that moderate AR gain in a TMPRSS2:ERG fusion-positive primary prostate cancer might synergistically enhance the expression of ERG, which gives growth advantage to those cells with moderate AR gain. ERG expression beyond a certain threshold would convey castration resistance to the tumor cells, which in turn increases the AR copy number and expression to compensate for androgen deprivation, contributing to disease progression and metastasis. Future work is needed to further study the hypothesis and the prognostic utility of the three-marker FISH panel.

**Heterogeneity of Genetic Aberrations Detected by the Three-marker FISH Panel**

The genetic heterogeneity assessment among CRPC patients showed that the major variability were between-patient. Within a given CRPC patient, aberrations in metastatic tumors were generally consistent across tumors, which are congruent with the general notion that metastatic cancer cells originated from the primary cancer cells and, therefore, likely maintain the same genetic lesion. However, some primary tumors may differ from metastatic lesions (Figure 3). This observation supported previous findings which demonstrated that primary prostate cancer is multiclonal, but most prostate cancer metastases are likely monoclonal in origin [25,36]. Also, primary tumors in the CRPC patient population have been exposed to aggressive therapy, which over time could result in genomic alterations inconsistent with the original primary tumor. In addition, intra-tumor variation was evident by both the AR and PTEN markers, which showed greater heterogeneity from tumors at the prostate site than distant metastases. This does not negate the significant intra-patient protein expression observed in our previously reported studies [37]. These findings support the multifocal and possibly multiclonal nature of advanced stage prostate cancer, especially at the prostate microenvironment [38].
In summary, we evaluated both primary cancer patients and Met/CRPC patients for the presence of TMPRSS2/ERG rearrangements, AR gene copy number gain, and PTEN deletion using a three-marker FISH panel. Our panel detected highly recurrent genetic abnormalities that showed different distributions between primary prostate cancer patients and Met/CRPC cancer patients. These abnormalities occurred more frequently in Met/CRPCs, which represent more aggressive disease, when present in localized primary prostate cancer, would convey aggressive characteristics to these localized tumors. Therefore, our results support the prognostic potential of the three-marker FISH panel for risk stratification. FISH findings strongly correlated with the transcription levels and provided further insight in the interaction of these three gene related functional pathways. Tumor heterogeneity analysis demonstrated more inter-patient variability than intra-patient, and that the intra-patient tumor heterogeneity was mainly due to the deviation of the prostate site tumor from metastases. Future studies will focus on applying this panel to retrospective or prospective studies on untreated primary cancer patients and on CTC/DTC to test its ability to stratify patients and predict clinical outcome.

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Supporting Information

Table S1 Shows the mRNA expression results of AR and ERG of all Met/CRPC tumor samples used in this study. (DOCX)

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Author Contributions

Conceived and designed the experiments: XQ MF. Performed the experiments: XQ GR CF LG IC MF. Analyzed the data: XQ GR CF BFK EM CSH CP RV PSN MF. Contributed reagents/materials/analysis tools: EM CSH CP RV PSN MF. Wrote the paper: XQ MF BFK IC EM RV PSN. Critical review of the manuscript: GR CF LG CSH CP.

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