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ERG AND CHD1 HETEROGENEITY IN PROSTATE CANCER: USE OF CONFOCAL MICROSCOPY IN ASSESSMENT OF MICROSCOPIC FOCI

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Abstract

Background—Biomarkers predicting tumor response are important to emerging targeted therapeutics. Complimentary methods to assess and understand genetic changes and heterogeneity within only few cancer cells in tissue will be a valuable addition for assessment of tumors such as prostate cancer that often have insufficient tumor for next generation sequencing in a single biopsy core.

Methods—Using confocal microscopy to identify cell-to-cell relationships *in situ*, we studied the most common gene rearrangement in prostate cancer (*TMPRSS2* and *ERG*) and the tumor suppressor *CHD1* in 56 patients who underwent radical prostatectomy. Contingency tables and the chi-square test were used to determine associations between mutation status of the genes and tumor phenotype.

Results—Wild type *ERG* was found in 22 of 56 patients; *ERG* copy number was increased in 10/56, and *ERG* rearrangements confirmed in 24/56 patients. In 24 patients with *ERG* rearrangements, the mechanisms of rearrangement were heterogeneous, with deletion in 14/24, a split event in 7/24, and both deletions and split events in the same tumor focus in 3/24 patients. Overall, 14/45 (31.1%) of patients had *CHD1* deletion, with the majority of patients with *CHD1* deletions (13/14) correlating with *ERG*-rearrangement negative status ($p < 0.01$).

Conclusions—These results demonstrate the ability of confocal microscopy and FISH to identify the cell-to-cell differences in common gene fusions such as *TMPRSS2-ERG* that may arise independently within the same tumor focus. These data support the need to study complimentary approaches to assess genetic changes that may stratify therapy based on predicted sensitivities.

Keywords

Prostate Cancer; *ERG*; *TMPRSS2*; Heterogeneity; confocal microscopy; *CHD1*

Introduction

Efforts to develop targeted therapies based on genomic findings are complicated by the polygenic nature of drug resistance and genetic heterogeneity (1). In addition to the importance of assessment of heterogeneity between separate tumor foci, we hypothesize that the use of complimentary approaches that assess heterogeneity within a single microscopic focus have important implications for predicting targeted drug therapy response and resistance (2) (3). Therefore, we studied the genetic heterogeneity of cells in microscopic foci within single biopsy cores using a confocal microscopy approach to precisely define local cell-to-cell variation, using the commonly found *ERG* rearrangements in prostate cancer as a model. In fact, the characterization of prostate cancer genomic changes has identified *TMPRSS2-ERG* fusions as the most frequent chromosomal rearrangement that, with recent studies, has been found to be strongly associated with the presence of the chromodomain helicase DNA-binding protein 1 (*CHD1*) gene (4). As studies are underway to understand the functional significance of these findings, data supporting an association of *TMPRSS2-ERG* and sensitivity to DNA damage has already led to clinical trials to determine if agents targeting DNA damage have differential activity for tumors with *TMPRSS2-ERG* fusions (5). Similarly, studies of *CHD1* have demonstrated it to be a tumor suppressor with its deletion associated with high Gleason score and absence of *ERG* fusion (6) (7).

Taken together, the identification of these important genomic changes such as *TMPRSS2-ERG* fusion and *CHD1* deletion and the question of tumor heterogeneity supports a thorough analysis of these potentially treatment stratifying biomarkers in biopsy cores. As a complimentary approach to the current literature, we used FISH analysis with confocal microscopy to carefully assess *ERG* rearrangements and *CHD1* to assess local cell-to-cell clonal variation.

Materials and Methods

Patients and Tissues

Using the TMA slides and confocal microscopy, we analyzed a common prostate cancer specific gene rearrangements (*TMPRSS2-ERG* fusion; *CHD1* deletion) in neighboring single cells in a tumor focus. We evaluated 56 patients who underwent radical prostatectomy at the Rutgers Cancer Institute of New Jersey with assessment of grading, *ERG*, *TMPRSS2*, and *CHD1*, with institutional review board approval. Tumor foci were identified within prostate cancer cores by pathology, and subjected to Fluorescence In Situ Hybridization and Confocal Microscopy. The average age of patients was 59.1 years (range from 49 to 73).

Fluorescence In Situ Hybridization (FISH)

TMA sections (4 mm) were cut onto SuperFrostPlus glass slides. *ERG* gene rearrangement was assessed using break-apart fluorescence *in situ* hybridization assay as described previously (8) (9). Bacterial artificial chromosomes (BACs) were obtained from the BACPAC Resource Center (Oakland, CA, USA). For detection of *ERG* rearrangement and *TMPRSS2-ERG* fusion, we used the following probes: RP11-95I21 (Alexa Fluor® 555 -

labeled; 5' to ERG) and RP11-476D17 (Alexa Fluor® 488 -labeled; 3' to ERG) and RP11-35C4 (Alexa Fluor® 647 -labeled; 5' to TMPRSS2). For detection of CHD1 deletion we utilized a gene-specific DNA probe (RP11-432N19, Alexa Fluor®647-labeled) and reporter probe that corresponded to pericentromeric sequence on human chromosome 5 (RP11-929P16, Alexa Fluor® 555- labeled).

Confocal Microscopy

Labeled samples were scanned using a confocal microscope (Zeiss LSM 510 META, 100x objective). Image stacks of 300 nm z-step size were captured and analyzed using Imaris Software (Bitplane). Gene rearrangement and copy number were evaluated by scoring spots in morphologically intact, non-overlapping interphase nuclei. Assessment was made for *ERG*, *TMRSS2*, and *CHD1* only in nuclei well defined in which both chromosomes were identified for each gene (mean of 49 nuclei per tumor TMA core for *ERG* and *TMRSS2* and mean of 74 nuclei per core for *CHD1*) as described previously (10). Expression of *ERG* gene in prostate cancer cells were confirmed by IHC using a monoclonal rabbit anti-human ERG antibody (clone EP111, dilution 1:50, Dako).

Transcriptome Sequencing

Libraries were constructed using the Illumina TruSeq™ RNA Sample Prep Kit v2 (Cat. No. RS-122-2001) according to the manufacturer's protocol, and 100bp paired-end sequencing was performed on the Illumina HiSeq2500. Raw reads from an Illumina HiSeq2500 in fastq format were aligned to a "filter" reference using bowtie to remove adaptor/primer, poly-A, poly-T, rRNA, tRNA, snRNA, and mitochondrial sequence. Reads were then aligned using BWA (version 0.5.9) against the genomic reference sequence for Homo sapiens (Build 37.2). The Homo sapiens (Build 37.2) reference sequence and annotation were pulled from RefSeq database (<http://www.ncbi.nlm.nih.gov/refseq/>).

Statistical Analysis

Fisher's exact test was used to assess the correlation of ERG rearrangements and CHD1 status (Table 1) and correlation of ERG copy number changes in T2 compared to T3 tumors (Table 2). Assessment of ERG status and CHD1 deletion were compared to Gleason score using the standard Cochran-Armitage trend test and also using an exact version of this trend test, using a permutation test implemented in the "independence_test" function in the "coin" package (<http://www.jstatsoft.org/v28/i08/>). Both the standard trend test, which uses the function "prop.trend.test", and the permutation "coin" package, are software programs implemented in the R statistical system (<http://www.R-project.org>).

Results

Confocal Microscopy Identifies Heterogeneity of ERG Rearrangements in Tumor Foci

Prostate cancer was assessed in 56 patients treated at the Rutgers Cancer Institute of New Jersey. The status of *ERG* rearrangements in adjacent nuclei of cancer cells was assessed using a combination of break-apart assay for *ERG* gene and additional FISH probe for conformation fusion with *TMRSS2* gene within each nucleus by confocal microscopy. As shown in Figure 1A and 1B, the status of *TMRSS2-ERG* fusion was identified within each

single nucleus, and whether the mechanism of fusion was through either *ERG* deletion (Edel) or a split with translocation of the *ERG* gene (Esplit). Increased *ERG* copy number without associated fusion was also characterized, as shown in Figure 1C.

To assess for cell-to-cell clonal variation, multiple adjacent cells (mean of 49 nuclei) were visualized individually within each tumor focus. As shown in Figure 2A, 56 patients with localized prostate cancer were assessed by FISH and confocal microscopy with wild type *ERG* found in only 22 of 56 patients (39.3%); *ERG* copy number increased in 10 (17.9%), and *ERG* rearrangements confirmed in 24 (42.9%), of which 22 resulted in a *TMPRSS2-ERG* fusion. The remaining two with *ERG* rearrangement without *TMPRSS2-ERG* fusion included a case with a split of 3' and 5' *ERG* signals and no evidence of fusion between *ERG* and *TMPRSS2* gene (the distance between green and white signals was more than 2 μ m), and the other case represents joint deletion of an *ERG* and *TMPRSS2* loci. In the 24 patients with *ERG* rearrangements (Figure 2B), the mechanisms of rearrangement were variable, with deletion in 14/24 (58.3%), a split event in 7/24 (29.2%), and both deletions and a split event in the same tumor focus in 3/24 (12.5%) patients. More specifically, regarding actual well defined nuclei of the three patients with both deletions and a split event in the same focus (Figure 2B, Esplit&Edel), the first patient had a deletion and a split event in 46.3% and 39% respectively of 41 nuclei analyzed; the second had a deletion and a split event in 58.1% and 35% respectively of 62 nuclei analyzed; and the third patient had a deletion and a split event in 22.6% and in 63.1% respectively of 57 nuclei analyzed. Figure 3C shows a picture of an example of the heterogeneity in patients with both *ERG* deletions and *ERG* split events that occurred in adjacent cells of the same tumor foci and resulted in *TMPRSS2-ERG* fusions.

Assessment with H&E (Figure 3A) and ERG immunohistochemistry (Figure 3B) demonstrated ERG expression in all 22 analyzed cases with *TMPRSS2-ERG* gene fusion and absence of ERG expression in all 32 cases without rearrangements, including the 10 cases with *ERG* copy number gain. One case with joint deletion of one *ERG* and *TMPRSS2* locus was also ERG expression negative. The case with a split of *ERG* and *TMPRSS2* loci was ERG positive, probably because of the *ERG* fusion with another gene partner that might drive the *ERG* gene expression. Although only a descriptive assessment, mean staining intensity was 1.78, 1.41, and 2.0 (scored 1–3) with a mean of 77.1%, 82.4%, and 89.1% of cells staining in patients with ERG deletion only, ERG split events only, and patients with both deletion and split events in the same foci respectively.

CHD1 deletion is associated with lack of ERG rearrangements in clones within Foci

Given prior studies demonstrating the dependence of *ERG* rearrangements on *CHD1*, we assessed *CHD1* in patient tumor using FISH and confocal microscopy to precisely assess adjacent nuclei (mean nuclei assessed 74 per tumor core) for *CHD1* deletions and copy number changes. As shown in Figure 4, using a DNA probe that corresponds to the pericentromeric region on human chromosome 5 as a reporter (red), *CHD1* deletion (Figure 4B) and copy number changes (Figure 4C) can be accurately identified in adjacent nuclei. As reported in Table 1, *ERG* rearrangement was significantly associated with normal *CHD1* gene status or copy number gain (using Fisher's exact test, $P=0.000188$). Specifically, of 45 patients in which both *ERG* status and *CHD1* could be accurately assessed, 14/45 (31.1%) of

patients had *CHD1* deletion, with the majority of the *CHD1* deletions 13/14 (92.9%) correlating with *ERG*-rearrangement negative status (Table 1).

CHD1 and ERG relationship to Tumor Aggressiveness and Stage

As shown in Table 2, a correlation between *ERG* copy number gain with T2 vs. T3 stage tumors was noted ($p=0.04963$). In contrast, *ERG* copy number gain did not correlate with tumor aggressiveness defined by Gleason Score ($p=0.256$). *CHD1* deletion (Figure 5B, red bars) also had a trend to correlate with more aggressive disease defined by Gleason score that was not statistically significant in this small data set ($P=0.115$), possibly defining an aggressive subset of tumors without *TMPRSS2-ERG* fusion. Clearly these data are only hypothesis generating, given the small sample size and the relevance copy number variations will require further studies. Additionally, a descriptive assessment demonstrates even more complexity for efforts to define copy number with heterogeneity of *CHD1* copy number within a single foci of some patients. Figure 6 shows copy number variation within tumor foci from patients known to have complex *ERG* rearrangements (shown in Figure 6 for the subgroup of 24 patients with *ERG* rearrangement). As shown in Figure 6, copy number changes in *CHD1* are noted for patients with heterogeneity of deletion or split events, but not in the three patients with a combination of split events and deletion in the same focus. Figure 7 shows assessment of one patient noted to have an *ERG* copy number gain and *CHD1* copy number gain in one focus (foci 1 of Figure 7A and Figure 7B and C) and wild type *ERG* with *CHD1* deletion in another focus (foci 2 of Figure 7A and Figure 7D and 7E), despite no clear evidence of *CHD1* deletion by RNA sequencing, as shown in Figure 7F.

Discussion

We found *ERG* rearrangement heterogeneity in patients with localized prostate cancer assessed by FISH and confocal microscopy, as a complimentary approach to assess local cell-to-cell clonal variation. We found heterogeneity in the mechanism of *TMPRSS2-ERG* fusions with variability in *ERG* deletions or translocations. *CHD1* deletion, which occurred in approximately 1/3 of patients, correlated with a distinct subset of higher grade tumors without *ERG* rearrangements.

Using a careful assessment of individual nuclei with FISH and confocal microscopy in patients with localized prostate cancer, we found *TMPRSS2-ERG* fusion rearrangements occurred in 39%. These data are similar to prior studies that have generally found approximately 40–50% of patients with *TMPRSS2-ERG* fusions (3). Prior studies using FISH alone (without confocal microscopy to identify each specific nucleus) also found homogeneity of types of *ERG* rearrangements within a given focus, and only heterogeneity between individual separate tumor foci (8) (10). In fact, Mehra et al. found 10% of 43 patients with both deletions and split events in the same patient, but in separate foci (8). Extending these observations, using confocal microscopy along with FISH to precisely assess cell-to-cell variations in each single foci, we now found marked heterogeneity in copy number, deletions and translocations even within a single focus of tumor. In fact, using confocal microscopy, we identified 29% of patients with *ERG* rearrangement with

heterogeneity with either a variable number of deletions, variable number of split events, or a combination of split events and deletions in a single focus (Figure 2B).

In line with this hypothesis that tumors exhibit significant intra-focal heterogeneity, Minner et al. recently studied *ERG* expression by immunohistochemistry and found both inter-focal and intra-focal staining heterogeneity, with general overall concordance between *ERG* positivity and FISH detected *TMPRSS2-ERG* fusions (11). Mertz et al. also found heterogeneous staining of *ERG* by immunohistochemistry in biopsy cores in 10 to 15% of patients (12). Our study using FISH and confocal microscopy to carefully assess individual foci (intra-focal heterogeneity) extends the assessments by Minner and Mertz et al. that used immunohistochemistry, providing genetic mechanisms for the heterogeneity present by these prior studies, confirming clonal variability even within a single focus of tumor. Assessment of *ERG* by IHC was not additionally informative to genetic assessments by FISH and confocal microscopy, as general intensity and percentage of cells staining was similar regardless of the type of *ERG* rearrangement in this subset. Future correlations within larger numbers of patients may be helpful for future comparison of methodologies. Future assessments will also be important to determine if these clonal differences detected by FISH and confocal microscopy that would be present in a single focus of a single biopsy core meaningfully relate to drug resistance and prognosis (13).

Given prior studies that have demonstrated *TMPRSS2-ERG* fusion generation in specific clones is dependent on *CHD1*, a putative TSG that codes for an ATP-dependent chromatin-remodeling enzyme, we also assessed *CHD1* using FISH and confocal microscopy. Prior studies using FISH analysis with a threshold of 60% loss of *CHD1* signal to define deletion and centromere 10 probe as control demonstrated 8.7% of prostate tumors with deletion (6). We demonstrated *CHD1* deletion to occur more frequently in 14/45 (31.1%) patients, possibly due to different technical approaches. In contrast to the approach by Burkhardt et al., we used FISH as well as confirmation of actual nuclei with both chromosomes using confocal microscopy and a chromosome 5 centromere probe (*CHD1* is located on 5q21) vs. Burkhardt who used a chromosome 10 control. Given the importance of *CHD1* and frequent finding of deletions in this small cohort, our data along with those by Burkhardt et al. warrants assessment in larger studies in which long term outcome is measured. Our data also demonstrated that *CHD1* deletion correlated with both a lack of *ERG* rearrangement and a non-significant trend with Gleason score, along with heterogeneity in copy number changes within foci. Although these data are only hypothesis generating given small numbers, these data are in agreement with prior studies that have also demonstrated that *CHD1* regulates genomic stability and its deletion is associated with high Gleason score and absence of *ERG* fusion (3) (6) (7). Liu et al. also demonstrated that *CHD1* is a tumor suppressor gene and knockdown has resulted in increased invasiveness (14). Baca et al. identified translocations and deletions to occur in an interdependent manner, or chromoplexy, coupled to transcriptional processes in tumors with *ETS* fusions; in contrast, tumors with *CHD1* deletion were associated with tumors without *ETS* fusions and a distinctive patterns of genomic instability, or chromothripsis (7). Given the association of *CHD1* and *ERG* status, as well as implications to tumor behavior in our study and these prior studies, larger

prospective studies with *CHD1* assessment are warranted to understand its role and mechanism as a predictive bio-marker.

Conclusions

Taken together, the identification of these important genomic changes such as *TMPRSS2-ERG* fusion and *CHD1* deletion and the question of tumor heterogeneity supports a thorough analysis of these potentially treatment stratifying biomarkers in even single tumor foci with few cancer cells in a biopsy core, in addition to efforts to attempts at multiple biopsies. These results also support the notion that *TMPRSS2-ERG* gene fusions may arise independently within the same tumor focus and that additional heterogeneity may be present even in a focus of a single biopsy core. Moreover, complex pattern of *ERG* and *CHD1* genes represents regional intra-tumor heterogeneity. These data support the study of complimentary approaches with NGS especially in tumors such as prostate cancer in which there is often limited tissue available. These data also require further study to understand heterogeneity of genetic changes, especially because clinical studies have already been launched, or are being designed, using genetic changes such as in a single core biopsy to direct a specific therapy.

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Figure 1. FISH and confocal microscopy assessment of *ERG* rearrangements

For detection of *ERG* rearrangements and *TMPRSS2-ERG* (T-E) fusion, we utilized multicolor interphase FISH and confocal microscopy. The intact *ERG* allele is designated by juxtaposition of red and green signals. The T-E gene fusion through deletion (Figure 1A: Edel, T-E fusion) was detected as the absence of a red signal and juxtaposition remaining green (3' to *ERG*) and white (*TMPRSS2*) signals. The split of red and green signals represents rearrangement of *ERG* gene through split and translocation (Figure 1B: Esplit, T-E fusion). Copy number gain of *ERG* gene represent polysomy of intact *ERG* and *TMPRSS2* alleles (Figure 1C).

Figure 2. Results of assessment of *ERG* rearrangements

Figure 2A: Distribution of *ERG* status in 56 patients. *ERG* wild-type (wt) was found in 22, *ERG* copy number gain in 10, and *ERG* rearrangement in 24 patients, with 22 patients demonstrating *TMPRSS2-ERG* fusion (E-T fusion). Figure 2B: *ERG* rearrangements occurred in 24 of 56 patients. The most frequent mechanism of rearrangements in micro-foci were deletion in 14/24 (58.3%), a split event and translocation in 7/24 (29%), and a heterogeneous mix of *ERG* deletions and translocations in 3/24 (12.5%) of patients.

Figure 3. Heterogeneity of *ERG* rearrangement

Figure 3A: FFPE sections of prostate cancer stained for AMACR expression (magenta) and expression of p63 (brown), which stains benign basal cells of the prostate. Figure 3B: Expression of ERG (brown), which was associated T-E fusion. Figure 3C: FISH analysis demonstrating intra-tumor heterogeneity with a combination of *ERG* deletion and *ERG* split/translocation within the same tumor focus.

Figure 4. Assessment of *CHD1*

FISH assay that included the specific BAC probe for *CHD1* gene (White) and the probe-reporter of the pericentromeric region of human chromosome 5 (Cen5), (Red). *CHD1* wild type (Figure 4A), deletion (Figure 4B), and increased copy number (Figure 4C) are shown.

Figure 5. Correlation of *ERG* and *CHD1* status with Tumor aggressiveness
ERG (Figure 5A) and *CHD1* (Figure 5B) rearrangement status is shown relative to tumor Gleason score with low (Gleason Score 6), intermediate (Gleason Score 7), and high aggressiveness (Gleason Score 8–10).

Figure 6. Variation of *CHD1* copy number in patients with complex *ERG* rearrangements
 The subgroup of 24 patients with *ERG* rearrangement are shown with associated assessment of *CHD1* (mean of 74 nuclei assessed per tumor core). Copy number changes in *CHD1* are noted for patients with heterogeneity of deletion (three graphs on left demonstrating percentage of nuclei with various copy numbers) or split events (single bar graph demonstrating most nuclei with 3 or more copies of *CHD1*), but not in the three patients with a combination of split events and deletion in the same focus.

Figure 7. Assessment of *ERG*, *CHD1*, and RNA sequencing

Figure 7A: Region 1 represents a focus of cancer cells with combination *ERG* gene and *CHD1* gene copy number gain, while in region 2 the cells with intact *ERG* alleles and mono-allelic deletion of *CHD1* gene. Figure 7B and C: H&E and FISH of focus 1, demonstrating increased *ERG* and *CHD1* copy number. Figures 7D and E: H&E and FISH of focus 2, demonstrating wild type *ERG* and *CHD1* deletion. Figure 7F: RNA sequencing using microdissection of multiple foci of tumor tissue demonstrating lack of recognition of heterogeneity of foci, with differences in *CHD1* in second focus.

Table 1

Relationship of ERG and CHD1 gene status in patient tumor assessed by FISH and confocal microscopy

		ERG		
		Wild type	Copy number gain	Rearranged
CHD1	Wild type	7	1	13
	Copy number gain	0	3	7
	Deletion	9	4	1

Table 2

Relationship of CHD1 and ERG status with pathological stage*

	CHD1 wt	CHD1 copy	CHD1 del		ERG wt	ERG copy	ERG rearrang
pT2a unilateral	1	1	0	pT2a unilateral	0	0	2
pT2c bilateral	12	3	6	pT2c bilateral	11	1	11
pT3a extra prostatic extension	7	4	7	pT3a extra prostatic extension	11	7	8
pT3b seminal vesicle invasion	0	1	2	pT3b seminal vesicle invasion	0	2	2

* Number of Patients with identified genomic status in each pathological stage