Title: Integrated Analysis Reveals Critical Genomic Regions in Prostate Tumor-Microenvironment Associated with Clinico-pathologic Phenotypes

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Running Title: Integrated genome-wide analyses for prostate cancer-stroma

Key Words: Prostate tumor-stroma, LOH, clinico-pathologic correlates
ABSTRACT

Purpose: Recent studies suggest that tumor microenvironment (stroma) is important in carcinogenesis and progression. We sought to integrate global genomic structural and expressional alterations in prostate cancer epithelium and stroma and their association with clinico-pathologic features.

Experimental Design: We performed a genome-wide loss-of-heterozygosity/allelic-imbalance (LOH/AI) scan of DNA from epithelium and stroma of 116 prostate cancers. LOH/AI hot- or cold-spots were defined as the markers with significantly higher or lower LOH/AI frequencies compared with the average frequency for markers along the same chromosome. These data were then integrated with publicly-available transcriptome datasets and our experimentally-derived data. Immunohistochemistry on an independent series was utilized for validation.

Results: Overall, we identified 43 LOH/AI hot-/cold-spots, 17 in epithelium and stroma (p<0.001), 18 only in epithelium (p<0.001), and 8 only in stroma (p<0.001). Hierarchical clustering of expression data supervised by genes within LOH/AI hot-/cold-spots in both epithelium and stroma accurately separated samples into normal epithelium, primary-cancer, and metastatic-cancer groups, which could not be achieved with data from only epithelium. Importantly, our experimental expression data of the genes within the LOH/AI hot-/cold-spots in stroma accurately clustered normal-stroma from cancer-stroma. We also identified 15 LOH/AI markers that were associated with Gleason score, which were validated functionally in each compartment by transcriptome data. Independent immunohistochemical validation of STIM2 within a stromal significant LOH marker (identified as associated with Gleason grade) confirmed its down-regulation in the transition from moderate to high Gleason grade.
Conclusions: Compartment-specific genomic and transcriptomic alterations accurately distinguish clinical and pathological outcomes, suggesting new biomarkers for prognosis and targeted therapeutics.

Abstract Word Count: 249
Translational Relevance

Genome-wide loss-of-heterozygosity/allelic-imbalance (LOH/AI) analysis of DNA from epithelium and stroma of 116 prostate cancers revealed 43 LOH/AI hot-/cold-spots, 17 in both epithelium and stroma (p<0.001), 18 only in epithelium (p<0.001), and 8 only in stroma (p<0.001). Integration of experimentally-derived transcriptome data of genes within the significant LOH/AI hot-/cold-spots in stroma revealed 15 LOH/AI markers that were associated with Gleason score. Independent immunohistochemical validation of one of these identified genes \textit{STIM2} within a stromal significant LOH marker (identified as associated with Gleason grade) confirmed its down-regulation in the transition from moderate to high Gleason grade. Stromal genomic and transcriptomic alterations may be helpful in pinpointing aggressive prostate cancers.
INTRODUCTION

Previous studies have mainly focused on changes in the epithelium, but recent investigations have highlighted the tumor microenvironment as equally important in carcinogenesis and progression. Genetic changes in tumor stroma have been reported by several independent groups working on a range of solid tumors (1-19), although typically, only selected markers have been used in most of these studies to assess loss-of-heterozygosity (LOH)/allelic imbalance (AI).

In prostate cancer (CaP), it has also been suggested that tumor microenvironment plays an important role in the progression, acquisition of androgen independence, and distant metastases (20-22). Tumor-microenvironment interactions through diffusible soluble factors such as transforming growth factor-beta (TGFB), as well as the extracellular matrix (ECM), likely lead to the development of metastasis (20). Nonetheless, little is known about the genetic basis of the human prostate tumor microenvironment; one report described genotypic heterogeneity in mesenchymal cells with a small number of markers (9) and another demonstrated allelic losses in the stromal component with a small number of samples and markers (23). These findings suggest that genetic alterations in CaP stroma exist and may be biologically relevant.

To attain a global perspective of epithelial- and stromal-specific LOH/AI changes for CaP and further refine our understanding of the LOH/AI regions, we performed a genome-wide LOH/AI scan using 381 microsatellite markers, and subsequently integrated these structural data with publicly available and experimental expressional array analyses. We also sought to determine if compartment-specific LOH/AI could be correlated with clinico-pathologic
features. Finally, we performed two types of validation: functional genomic validation and immunohistochemistry in an independent series of CaP.

METHODS

Patients and Samples

We obtained 116 CaPs at any tumor classification with or without lymph node metastasis and analyzed them in accordance with the respective Institution’s Human Subjects Protection Committees. All slides were re-reviewed by a single genito-urinary pathologist (P.Z.). Clinical information such as Gleason score, tumor volume, and the status of lymph node metastasis was noted (Suppl Table 1). An independent validation series of CaP are described under Immunohistochemical Analysis.

Laser Capture Microdissection (LCM) and Genome-wide LOH/AI Scan

LCM of non-neoplastic normal tissue, epithelial carcinoma cells and tumor-associated stroma was performed from formalin-fixed paraffin-embedded tissue for all 116 cases, as previously described (7, 8, 17, 24, 25) and demonstrated at the link http://content.nejm.org/cgi/content/full/357/25/2543/DC2. Stromal fibroblasts resided in between aggregations of epithelial tumor cells or no more than 0.5 cm distant from tumor nodule. While epithelial-stromal cell cross-contamination is a possibility, we utilized standard and well-established protocols to minimize this, as reported previously (8, 18, 24, 25). Corresponding normal DNA for each case was procured from normal tissue, located a distance away from the tumor or from a different tissue (formalin-fixed paraffin-embedded) block containing only normal tissue (latter first choice). Genomic DNA was extracted as previously
described, with the exception that incubation in proteinase K was done at 65°C for two days (7). Primer sets for multiplex PCR defined 381 microsatellite markers in 72 multiplex panels (Research Genetics, Invitrogen). Genotyping, analyses, and scoring of LOH/AI were done as reported (24, 26, 27). The methodological veracity of LOH/AI scan using multiplex-PCR on archived templates was extensively validated as published, i.e., by a series of control experiments using chromogenic in situ hybridization (CISH), quantitative PCR, etc., to eliminate the possibility of epithelial-stromal cell cross-contamination and to exclude any possibility of PCR artifact (24, 25).

Data Analysis

The dataset contains LOH/AI status at 381 microsatellite markers for 116 different samples, each with neoplastic epithelium, tumor stroma and normal tissue. The same 10 markers were never informative in at least one compartment and so excluded from further analyses. First, we sought to define regional LOH/AI hot-/cold-spots as the markers with significantly higher or lower frequency of LOH/AI compared with the average frequency of the markers along the same chromosome. We analyzed hot- and cold-spots of LOH/AI together with the concept that the genetic endpoint may be similar, e.g., loss of a tumor suppressor gene and gain (cold spot) of an oncogenic event. Toward those ends, for each informative marker, the statistical significance of overall (across all samples) LOH/AI frequency compared with the respective chromosome average was analyzed using the exact binomial test (R base package binom.test; http://www.r-project.org). Second, the associations of LOH/AI across all informative markers (not just hot/cold-spot markers) in epithelial and stromal samples with presenting clinico-pathologic features (i.e., Gleason score, tumor volume, and the status of lymph node...
metastasis) were analyzed using a logistic model with LOH frequency as an outcome and each clinico-pathologic feature as a predictor (28, 29). Multiple testing adjustment was applied by controlling false positive report probability (FPRP) (30) with a prior probability of 0.05 or 0.01. The FPRP indicates the probability that a statistically significant finding is a false positive by considering 3 factors: the $P$ value magnitude, the statistical power, and the prior probability of true associations. Only those with $P$ values $<$ 0.05 and estimated FPRP values less than 50%, indicating a small probability of being a false positive, are reported as statistically significant findings. An FPRP value with a prior probability of 0.05 and less than 50% is denoted as FPRP$_{0.05}$$<$ 0.5. We used more strict criteria, FPRP$_{0.01}$$<$ 0.1, for LOH/AI hot-/cold-spots expecting higher significant regions. For the association of LOH/AI with clinico-pathologic features, we applied FPRP$_{0.05}$$<$ 0.5. For the positive correlation of LOH/AI with Gleason score, $P$ value is obtained by one-side trend test (R, prop.trend.test) with false discovery rate (FDR) being controlled for correcting multiple testing.

Integration of Public Microarray Data with LOH/AI Data

Raw data from Gene Expression Omnibus (GEO) series GSE3325 at GEO platform GPL570 (Affymetrix HG-U133_Plus_2) were downloaded (31) and normalized using Robust Multi-array Averaging (RMA) (32). The GSE3325 data included 13 individual benign, primary and metastatic CaPs, and 6 pooled samples from benign, primary or metastatic CaPs. These specimens were grossly dissected maintaining at least 90% of the tissue of interest, based on examination of the frozen sections of each tissue block. We integrated microarray data of the genes residing within the regions that showed significant LOH/AI with our genetic data. We sought to utilize the expression microarray data in order to validate our LOH/AI hot-/cold-spots
and to identify the significant genes and pathways in prostate carcinogenesis or progression. Throughout this study, we consistently selected all genes, that are located within 250kb upstream and downstream of the LOH/AI regions of interest (significant hot/coldspots), that have significant expression values and that are included in each microarray dataset, for hierarchical clustering analyses. All hierarchical clustering analyses in this study were performed using Cluster and TreeView softwares (http://rana.lbl.gov/EisenSoftware.htm) (33). Genes belonging to hot-/cold-spots in both epithelium and stroma or in only epithelium were selected and hierarchical clustering was conducted to both genes and samples.

**Generation of Prostatic Stroma Microarray and Integration with LOH/AI Data**

We generated stromal microarray expression data for functional validation of our LOH/AI hot-/cold-spots in stroma because we could not find appropriate public microarray datasets for prostatic stroma. Stromal cells cultured from normal peripheral zone tissues (F-PZ-64, F-PZ-79, F-PZ-82, F-PZ-102, F-PZ-105) and from tumors (F-CA-31, F-CA-39, F-CA-52, F-CA-67, F-CA-93) were established and grown as previously described (34). Total RNAs were extracted from semi-confluent cells (passages 4-5) one day after feeding fresh medium using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions and treated with TURBO DNA-free kit (Ambion). Hybridizations were performed according to Illumina protocols by the Genomic Core, Lerner Research Institute, Cleveland Clinic. All samples were hybridized to the Illumina Sentrix Human-6_v3 Expression BeadChip, which contains 48,000 distinct oligonucleotide probes. These transcriptome data are deposited in GEO (GSE34312 [not publicly released yet]). After normalization using average normalization algorithm of BeadStudio Gene Expression Module v3.4 (Illumina), we selected all the genes within +/- 250
kb of LOH/AI hot-/cold-spots in only stroma and applied a hierarchical clustering method to both genes and samples.

**Integration of Microarray data with LOH/AI Regions Associated with Clinico-pathologic Features**

We selected all the genes lying within and +/-250 kb of the significant LOH/AI markers associated with Gleason score or tumor volume in both epithelium and stroma. With the genes associated with Gleason score or tumor volume, a hierarchical clustering was performed for epithelium using public microarray data, GSE3325 and for stroma using our stromal microarray data (31).

**Immunohistochemical Analysis**

Formalin-fixed and paraffin-embedded CaP sections from 49 independent cases were immunostained with a rabbit anti-STIM2 polyclonal antibody (Abcam Inc., Cambridge, MA) for STIM2 expression in CaP stroma. Both Gleason grade 3 (G3) and Gleason grade 4 and/or 5 (G4/5) lesions were examined for each case. Deparaaffinized tissue sections were placed in 10 mM citrate buffer (pH 6.0) and were heated to 125°C in a Decloaking Chamber (Biocore Medical, Concord, CA) for 30 seconds for antigen retrieval. These sections were then incubated with a 1:300 dilution of primary antibody overnight at 4°C, secondary antibody and the avidin-biotin complex (VECTASTAIN Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA), and developed with diaminobenzidine. Sections were counterstained with hematoxylin. For negative controls, primary antibody was omitted. Stromal expression of STIM2 was determined by counting sum of intensities of positive stromal cells divided by area
of stroma, using Image-Pro Plus 7.0 (Media Cybernetics, Inc., Bethesda, MD). Five random fields were analyzed with a magnification of 40x. STIM2 expression in tumor stroma was divided by those in normal stroma for normalization. The statistical significance of STIM2 expression levels between G3 and G4/5 lesions was determined with Wilcoxon sign rank test.

RESULTS

LOH/AI Hot-/cold-spots in CaP
Overall, 371 markers across all chromosomes, ranging from 7 on chromosome 22 to 31 on chromosome 1, were analyzed: 38,460 PCR-reactions (19,639 for epithelium, 18,821 stroma) were informative for LOH/AI evaluation. We identified a total of 43 hot-/cold-spots, 17 occurring in both epithelium and stroma, 18 only in the epithelium, and 8 only in the stroma (Table 1). No known genes were contained at 7 loci (D1S2134, D3S2427, D4S2417, D6S1277, D9S922, D12S2078, and D14S606), however, we found ESTs at these loci (Table 1). Genes belonging to hot-/cold-spots common to both epithelium and stroma include HOXB1-HOXB9 cluster, HOXB13, ZNF554, ZNF555, ZNF556, ZNF57, and ZNF77 as well as 7 miRNA (Table 1). FGF12, ACVRIB, the KRT cluster and APOL1-APOL4 as well as 6 miRNAs were included in the genes lying in hot-/cold-spots of LOH/AI in epithelium only (Table 1), and TGFBIII, WFDC5, WFDC12 and TP53TG5 as well as 5 non-coding RNAs, notably miR-125b-1, were among the genes of hot-/cold-spots in stroma only (Table 1).

Association of LOH/AI with Presenting Clinico-pathologic Features
LOH/AI markers in association with clinico-pathologic features (Suppl Table 1) may reveal clues to clinical behavior and biological diversity and/or serve as biomarkers of prognosis or
therapeutic options. Using logistic regression, we analyzed our genome-wide LOH/AI scan across all informative markers in order to identify compartment-specific loci that were significantly associated with aggressiveness of disease as reflected by Gleason score, tumor volume, and regional nodal status. We identified 15 LOH/AI regions, 10 in epithelium and 5 in stroma, associated with Gleason score (Table 2), 11 (8 in epithelium and 3 in stroma) of which showed a positive correlation with increasing Gleason score (one-sided trend test, FDR<0.05; Suppl Table 2). Among these 11 markers, 8 (6 in epithelium and 2 in stroma) remained significantly positively correlated with Gleason score even when a more rigorous method was applied (Bonferroni adjusted p<0.05). In association with tumor volume, 40 markers were identified in epithelium and 35 in stroma (Suppl Table 3). Interestingly, there were no significant LOH/AI markers correlated with regional nodal metastases.

Expression Profiling of Genes within LOH/AI Hot-/cold-spot Regions Using Publicly Available Neoplastic Epithelial Microarray Data

We focused on genes of LOH/AI hot-/cold-spot regions and analyzed their expression profiles using publicly available microarray data (see METHODS). This served two purposes. First, this acts as a functional (transcript-expressional) validation of the genetic data. Second, integration of the genetic and expressional data would lend clues to the compartment-specific functions of the genes within the hot-/cold-spot regions as they relate to progression and prognosis. Hierarchical cluster analysis supervised by 87 genes found in LOH/AI hot-/cold-spot regions +/-250kb resulted in 3 distinct groups: normal epithelium, cancer, and metastatic cancer when considering genetic information from both epithelium and stroma (Figure 1A). When we used 61 genes residing within the LOH/AI hot-/cold-spot +/-250kb regions occurring
specifically in epithelium, only two groups could be clearly distinguished, namely, normal epithelium and metastatic cancer groups (Figure 1B). Interestingly, the primary cancer samples were equally clustered with normal epithelium or with metastatic disease.

**Expression Profiling of Genes within LOH/AI Hot-/cold-spot Regions Using Our Experimentally Generated Stromal Microarray Data**

We performed a hierarchical clustering of stromal cells, supervised by 28 genes of LOH/AI hot-/cold-spot +/- 250kb regions from stroma only (Figure 2). This accurately separated normal stromal cells and cancer-derived stromal cells.

**Expression Profiling of Genes within LOH/AI Regions Associated with Clinico-pathologic Features**

We further focused on genes residing in proximity to our LOH/AI regions associated with Gleason score and tumor volume, and analyzed their expression profiles using publicly available microarray data (see METHODS) for epithelial analysis as well as our experimentally generated expression data for stromal analysis. With only one exception, P3, in neoplastic epithelial analysis, samples were clearly separated into normal, primary cancer, and metastatic disease by 31 genes within the LOH/AI (+/- 250kb) regions in neoplastic epithelium associated with Gleason score (Suppl Figure 1A). Our stromal cell cultures derived from normal tissues and from CaP were accurately clustered supervised by the 18 genes belonging to our stroma-only LOH/AI (+/- 250kb) regions associated with Gleason score (Suppl Figure 1B). As for tumor volume, epithelial CaP samples were also successfully separated into normal, primary cancer and metastatic disease in epithelial analysis (Suppl Figure 2A). In contrast, our stromal
cell cultures did not cluster into tumor-derived stroma and normal stroma groups in stromal analysis for tumor volume (Suppl Figure 2B).

**Independent Validation of Candidate Gene by Immunohistochemical Analysis**

To further validate our genetic data, we selected our strongest candidate gene, *STIM2*, lying in D4S2397, which is the most significant stromal-associated LOH marker correlated with Gleason score (Suppl Table 2). We examined STIM2 expression in 49 independent primary CaP samples that had both Gleason grade 3 and Gleason grade 4 and/or 5 lesions by immunohistochemical analysis (Figure 3). Stromal protein expression of STIM2 (Y-axis) showed significant down-regulation in the stroma of G4/5 lesions compared to that of G3 lesions (p<0.001; Figure 3A).

**DISCUSSION**

Recent molecular studies have provided a myriad of information about genetic changes in CaP and accumulating data over the last two decades have shown the importance of the tumor stroma in prostate carcinogenesis, with the latter based on mainly functional and cell biological studies. Here, we have completed a genome-wide LOH/AI scan and integrated these data with global expression array data for CaP epithelium and stroma, and correlated these with presenting clinico-pathologic features, and validated our observations via functional genomics approach and immunohistochemistry of a newly identified stromal-relevant gene associated with Gleason score.

First, genome-wide LOH/AI scan revealed LOH/AI hot-/cold-spots that were important in each compartment alone and in both compartments. Among the 225 genes in proximity to
the 43 LOH/AI hot-/cold-spot regions germane to epithelium and/or stroma, several candidate tumor suppressor genes, such as \textit{HOXB13} (epithelium and stroma), the \textit{APOL1-4} cluster and \textit{ACVR1B} (epithelium), and \textit{TP53TG5} (stroma), are notable. The \textit{APOL} family of genes (\textit{APOL1-6}) encodes programmed cell death proteins that can initiate apoptosis or autophagic cell death, as well as belong to a multi-component innate immunity complex-building platform (35). Thus, loss-of-function of the \textit{APOL1-4} region will lead to loss of apoptosis and autophagic cell death regulation as well as potential decreased innate immunity locally. Our observations here, ie, loss of APOL1-4 function leading to loss of autophagic cell death and consequent loss of essential nutrients and energy to tumor epithelial cells in a paracrine fashion are consistent with the autophagic tumor stroma model of cancer metabolism underscored by Lisanti’s body of work (36). Furthermore, the relevance to innate immunity lends credence to early hypotheses of metabiomic relationships in initiating various neoplasias (35, 37), including that of the prostate. Of note, the activin-TGFB signaling sub-network has already been acknowledged to be important in prostate carcinogenesis (38). We have shown that D12S297 harbors the human activin receptor, \textit{ACVR1B}, which has been described as having somatic mutations and deletions in at least 4 other types of cancers, and which is expressed in prostate epithelium (39). \textit{ACVR1B} encodes an activin receptor that signals via the TGFB pathway as well.

Second, we found 15 specific loci of LOH/AI in the epithelium or stroma associated with Gleason score (Table 2). We suspect that these regions and genes are relevant to invasion and progression and focused especially on stroma. Two loci (4p15.2 and 19p13.3) harbored significant LOH/AI in the stroma, correlated with Gleason score. One of the genes located in the 4p15.2, which is the most significant region, is STIM2 (stromal interaction molecule 2).
Our immunohistochemical analysis of 49 independent CaP samples has given us an in-principle validation of our genetic and functional-genomic studies. STIM1 was originally proposed as a candidate tumor suppressor and regulates intracellular calcium (40, 41). STIM2 is also a transmembrane protein, that, in limited studies, has been shown to mediate cellular proliferation (42). In view of our observations, further studies on the role of STIM2 in different cancers, especially in different compartments, are required. The 19p13.3 region contains AES which is known to be associated with Wnt signaling pathway and MAPK/ERK1, which is an upstream kinase for NF-kappaB activation (43, 44). The cluster of ZNFs including ZNF77 also at the 19p13.3 region was previously associated with different cancers, and ZNF77 status has been used as a prognostic marker in early stage renal cell carcinomas and stage I breast cancers to predict recurrence (45, 46).

We subsequently integrated global transcriptome data from public archives and those we experimentally derived with our LOH/AI hot-/cold-spots. Importantly, these provided functional validation of our genomic data. The gene expression profile using neoplastic epithelium from public sources with only genes from our epithelium-related LOH hot-/cold-spots, which is not the entire epithelium gene expression profile, could not accurately cluster the samples. Similarly, we also attempted the cluster analysis supervised by the genes from only stromal hot-/cold-spots, and as expected, it resulted in failure to cluster the samples (data not shown). In contrast, these samples could be accurately clustered when taking into account the genes from the stromal hot-/cold-spots, indicating that genomic alterations with consequent expressional alterations in the stroma are necessary and play an important role in prostate carcinogenesis and progression. Equally important is a notable negative. The genes within the LOH hot/coldspot regions identified as important in correlating with tumor volume were
germane only in the epithelium. This was validated when expression data supervised by the
genes within the LOH hot/coldspot regions relevant to tumor volume could not accurately
classify normal stroma from tumor stroma (Suppl Figure 2B). In other words, at least in CaP,
tumor volume does not appear to be influenced by stromal genomic or expressional alterations
of genes located within tumor volume-associated stromal LOH regions in this study. This
makes teleological logic given that the genes playing roles in tumor stroma are almost certainly
relevant to invasion, progression and metastasis, as noted above.

By integrating genomic and global expression data for the epithelium and stroma of
CaP, we were able to detect significant regions and candidate genes important in the tumor-
microenvironment crosstalk that leads to carcinogenesis and progression. Importantly, we have
performed functional genomic validation of the genes in the regions associated with Gleason
score as well as tumor volume, in a compartment-specific manner, as well as validated a
stromal gene associated with Gleason score in an independent series of tumors using a different
technique, namely, immunohistochemistry for STIM2, as proof of principle. Parenthetically,
we also noted that the epithelial expression of STIM2 (D4S2397 is also a significant epithelial-
associated LOH marker correlated with Gleason score as well) was down-regulated in G4/5
over G3. However, we did not formally analyze the epithelial expression in this study because
the effect size did not appear as marked as for stroma. These data provide fundamental insights
into compartment-specific roles in prostate carcinogenesis or progression and may also reveal
the solid tumor microenvironment as a novel compartment for important biomarkers of
prognosis as well as targeted therapy.
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FIGURE LEGENDS

Figure 1. Dendrogram and heatmap of hierarchical clustering analysis using 19 prostatic samples from public microarray database.
A, Supervised by 87 genes within LOH/AI hot-/cold-spots +/- 250 kb in epithelium and stroma. In the sample axis, samples were clearly separated into three classes. In the gene axis, the genes were clustered in different branches according to similarities in relative expression. B, Supervised by 61 genes within LOH/AI hot-/cold-spots +/- 250 kb in only epithelium. Note that supervision only by the genes within epithelial hot-/cold-spots resulted in the primary tumors not being able to be classified correctly and were instead sorted equally as normal prostate or metastatic disease. This is in contrast to A where genomic information from both epithelium and stroma was integrated with expression array data. N, benign prostate; P, primary CaP; W, metastatic CaP samples; NX, PX, or WX, respective mixtures of benign, primary, or metastatic CaP samples (i.e. NX1 indicates the mixture of N1-4 and NX2 is the duplicate of NX1). Red, the transcript level is above the median for that gene across all samples; green, below the median for that gene across all samples; black, unchanged expression; gray, no detectable expression.

Figure 2. Dendrogram and heatmap of hierarchical clustering analysis supervised by 28 genes within stromal LOH/AI hot-/cold-spots +/- 250 kb across 10 stromal cell cultures derived from normal tissues (F-PZ) or tumors (F-CA).
Note that cell cultures were accurately clustered into each group according to origin. VKORCl and ROCK2 that appear twice and show different profiles represent probe sets that recognize different splice isoforms. Colors, sample names, and the other conditions are the same as those of Figure 1.

Figure 3. Immunohistochemical Analysis reveals decreased STIM2 expression in the stroma of Gleason grade 4 and/or 5 (G4/5) regions compared to that of Gleason 3 regions.
A, The evaluation in immunohistochemical staining of CaP stroma with anti-STIM2 antibody, indicating that STIM2 gene expressions were significantly down-regulated in G4/5 in comparison with G3 (Wilcoxon sign rank test, p<0.001). X axis shows Gleason grade and Y axis indicates stromal expression of STIM2 (see METHODS). B, Representative staining lesions of G3 and G4/5 for the same case are shown (400x).
Table 1. LOH/AI Hot-/cold-spots in Prostate Cancer

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<th>Markers</th>
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<td>&lt;0.001</td>
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<td>D17S2180</td>
<td>17q21.32</td>
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<td>SKAP1, HOXB1 through HOXB9, HOXB13, PRAC2, C17orf92, TTLL6</td>
<td>mir-10a, mir-196-1, mir-152</td>
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<tr>
<td>D19S591</td>
<td>19p13.3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>ZNF554, ZNF555, ZNF556, ZNF57, ZNF77, TLE2, TLE6, AES, GNA11</td>
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<td>D20S103</td>
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<td>TBC1D20, CSNK2A1, TCF15, SRXN1</td>
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In Epithelium Only

<table>
<thead>
<tr>
<th>Markers</th>
<th>Loci</th>
<th>Ep</th>
<th>Genes (within ±250kb of markers)</th>
<th>miRNAs (within ±2Mb)</th>
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<td>D1S1612</td>
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### In Stroma Only

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<th>Genes (within ±250kb of markers)</th>
<th>miRNAs (within ±2Mb)</th>
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<td>GRAMD1B, SCN3B, ZNF202, OR6X1, OR6M1</td>
<td>mir-26b, mir-153-1, mir-100, let-7a-2, mir-125b-1</td>
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<td>D11S4464</td>
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<td>PMP22CD, OR8D4, OR4D5, OR6T1, OR10S1</td>
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<td>D12S1045</td>
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<td>D20S481</td>
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*a* Multiple testing adjustment is based on FPRP0.01<0.1
Table 2. LOH/AI in Epithelium and Stroma Associated with Gleason Score

### Gleason Score and LOH/AI in Epithelium

<table>
<thead>
<tr>
<th>Markers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Loci</th>
<th>ROH</th>
<th>LOH</th>
<th>ROH</th>
<th>LOH</th>
<th>ROH</th>
<th>LOH</th>
<th>P Value&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>1p36.23</td>
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<td>4p15.2</td>
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### Gleason Score and LOH/AI in Stroma

<table>
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<th>Marker</th>
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<th>LOH</th>
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<th>LOH</th>
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<th>LOH</th>
<th>P Value&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>4p15.2</td>
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<td>5</td>
<td>14</td>
<td>1</td>
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<sup>a</sup>Three markers, which are associated with Gleason score, were excluded because missing in more than half.

<sup>b</sup>Multiple testing adjustment is based on FPRP0.05<0.5

ROH, retention of heterozygosity; LOH, loss of heterozygosity

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