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Cells Comprising the Prostate Cancer Microenvironment Lack Recurrent Clonal Somatic Genomic Aberrations

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Abstract

Prostate cancer-associated stroma (CAS) plays an active role in malignant transformation, tumor progression, and metastasis. Molecular analyses of CAS have demonstrated significant changes in gene expression; however, conflicting evidence exists on whether genomic alterations in benign cells comprising the tumor microenvironment (TME) underlie gene expression changes and oncogenic phenotypes. This study evaluates the nuclear and mitochondrial DNA integrity of prostate carcinoma cells, CAS, matched benign epithelium and benign epithelium-associated stroma by whole genome copy number analyses, targeted sequencing of TP53, and fluorescence *in situ* hybridization. Comparative genomic hybridization (aCGH) of CAS revealed a copy-neutral diploid genome with only rare and small somatic copy number aberrations (SCNAs). In contrast, several expected recurrent SCNAs were evident in the adjacent prostate carcinoma cells, including gains at 3q, 7p, and 8q, and losses at 8p and 10q. No somatic TP53 mutations were observed in CAS. Mitochondrial DNA (mtDNA) extracted from carcinoma cells and stroma identified 23

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somatic mtDNA mutations in neoplastic epithelial cells but only one mutation in stroma. Finally, genomic analyses identified no SCNAs, no loss of heterozygosity (LOH) or copy-neutral LOH in cultured cancer-associated fibroblasts (CAFs), which are known to promote prostate cancer progression *in vivo*.

Keywords

prostate cancer; tumor microenvironment; copy number alterations; genomic aberrations; CGH array; stroma; fibroblast

INTRODUCTION

Organs in which carcinomas arise are complex systems comprised of distinct populations of resident and transitory cell types that are critical for the maintenance of specialized tissue functions. Following organogenesis, epithelial cells maintain a homeostatic relationship with mesenchymal components, collectively termed stroma, that include matricellular structural proteins and a spectrum of cell types such as endothelium, nerve cells, smooth muscle and fibroblasts. Beyond simply providing a static architecture, stromal elements form dynamic and reciprocal interactions with epithelium that are increasingly recognized to influence the development and progression of neoplastic growth (1,2).

Within prostate carcinomas, a marked change in the cancer-adjacent microenvironment is often observed. These changes, termed a reactive stroma, are notable for the loss of differentiated smooth muscle cells and the gain of myofibroblast and fibroblast cell types that are accompanied by an abundant extracellular collagen matrix. The extent of this reactive stroma has been shown to associate with adverse prostate cancer behavior and assessments of gene expression have demonstrated substantial changes in the transcriptional programs active in this tumor microenvironment (3). Functionally, prostate cancer-associated fibroblasts (CAFs) cultured from regions adjacent to cancer cells induce tumors when recombined with initiated, but non-tumorigenic, epithelial cells (4). Importantly, these CAFs maintain cancer-promoting effects even without continued exposures to cancer cells, suggesting that genetic and/or epigenetic alterations might underlie their cancer-promoting capabilities (reviewed in (5)). In this context, the genetic deletion of *TGFBR2* or *p62* in prostate fibroblasts has been shown to causally increase the frequency of prostate intraepithelial neoplasia (PIN) *in vivo* (6,7).

In view of the substantial microenvironment histological changes that accompany the development of prostate cancer, and the potent inductive power of prostate CAFs, several studies have been conducted to identify the underlying molecular basis for these alterations with the hypothesis that they may be influenced by underlying somatic genomic aberrations. A report evaluating three microsatellite markers on chromosome 8p reported that 3 of 9 patients with 8p LOH in the tumor cells also showed LOH in stroma (8). In a study of the rare phyllodes subtype of prostate cancer, SCNAs were identified by microsatellite analysis in both epithelial and stromal compartments suggesting that both elements are clonal and neoplastic. However, discordant LOH patterns were observed between epithelium and stroma indicating different clonal origins (9). A study evaluating 116 cases of primary

prostate cancer employed a panel of genome-spanning microsatellite markers to assess LOH/AI separately in the epithelium and stroma. Distinct aberrations were found in the different cell compartments with 8 markers demonstrating LOH specifically in the stroma of a subset of tumors (10).

In the present report we sought to further delineate the frequency and type of genomic aberrations in stromal cell constituents of prostate carcinomas in order to identify potential causal factors underlying microenvironment mediators of adverse cancer phenotypes.

EXPERIMENTAL METHODS

Prostate carcinoma samples and tissue microdissection

Twenty fresh-frozen prostate adenocarcinomas and adjacent benign tissue were obtained from Baylor College of Medicine and the University of Washington under approval from their respective Institutional Review Boards. Fifteen samples had a combined Gleason score (GS) of 7 and five had a GS>7.

To obtain tumor epithelium and tumor stroma, laser capture microdissection was performed. In brief, frozen sections (7 μ M) were cut from OCT embedded snap-frozen prostate tissue into PAP-membrane slides. Approximately 4000 cells were microdissected from four spatially and histologically-defined cell compartments: prostate cancer epithelium (CPE), benign prostate epithelium (BPE), prostate cancer-adjacent stroma (CAS), benign prostate epithelium-adjacent stroma (BAS). For CAS, we specifically captured stromal fibroblasts adjacent (<1 mm away) to neoplastic epithelium and avoided foci of inflammatory cells. The corresponding benign cells for each case were microdissected from separate blocks identified as containing no adenocarcinoma cells (first choice) or, from non-neoplastic tissues at a distance >1mm from the cancer. Digital photos were taken of tissue sections before, during, and after LCM and assessed to confirm the cell type-specificity of the captured cells.

For the preparation of cancer-associated fibroblasts (CAF) and benign/normal associated fibroblasts (NAF), de-identified prostate tissue samples were collected at Vanderbilt University under local IRB approval through the NCI-funded Cooperative Human Tissue Network. CAF and NAF were isolated from cancer and normal tissue respectively, as previously described (11). CAF activity was validated using a tissue recombination bioassay in which CAF or NAF were recombined with initiated reporter epithelium (BPH-1) and grafted to the renal capsule of severe combined immunodeficient (SCID) mice. Cells were classified as CAF if they demonstrated the ability to induce tumorigenesis (defined grossly and histologically) in the epithelial cells under these conditions. Cells from benign sources were also checked to confirm that they did not induce tumor growth.

DNA extraction and amplification

Genomic DNA was extracted from microdissected tissue using the QIAamp DNA micro kit (Qiagen, Inc.) according to the manufacturer's instructions, except that the samples were incubated in RLT buffer with proteinase K overnight at 56°C before DNA extraction. DNA concentrations were measured by Quant-iT™ dsDNA HS Assay Kit and read on a Qubit™

Fluorometer (Invitrogen). Aliquots of extracted DNA (10 ng) were amplified to yield sufficient DNA for aCGH using random fragmentation whole genome amplification following the manufacturer's instructions (WGA2, Sigma-Aldrich).

Array comparative genomic hybridization (aCGH)

We used the SurePrint G3Human CGH Microarray Kit 2×400K (Design ID 023364, Agilent Technologies) according to the manufacturer's protocol. In brief, 2 µg of WGA2-amplified DNA was labeled with Cy3 dye (in the case of experimental epithelial and stromal DNA) or Cy5 dye (in the case of male reference DNA, purchased from Promega, Part No. G1471) according to manufacturer's instructions. Labeled Cy3 and Cy5 DNA were combined and diluted in Agilent Hi-RPM Hybridization Buffer, Agilent Blocking agent and human Cot-1 DNA (Invitrogen). aCGH slides were scanned (Agilent DNA Microarray Scanner) at a resolution of 2 µm. All data were extracted from raw images and normalized using the Feature Extraction software version 9.5 (Agilent Technologies) to obtain Agilent FE data. Additional details are provided in Supplementary Extended Experimental Methods.

For the primary cancer-associated fibroblasts (CAF) and its counterpart normal prostate fibroblasts (NAF) prior to labeling, 2 µg of genomic DNA was digested for 2 hours at 37°C with 1unit/mL of both AluI and RsaI restriction endonucleases (Invitrogen). The reaction was terminated by incubating at 65°C for 20 minutes. Digested DNA was subsequently labeled as described above. The presence of DNA copy number aberrations and copy-neutral LOH was evaluated in 3 CAF/NPF sample pairs using the Infinium HumanOmniExpressExome BeadChip Kit (Cat No. WG-350-2206, Illumina Inc) according to the manufacturer's protocol using 100 ng of genomic DNA. The data is available in the Gene Expression Omnibus (GEO) database (accession GSE76456).

Array CGH data analysis

Nexus copy number analysis—Agilent FE data was analyzed using Nexus Copy Number version 6.0 software (BioDiscovery, CA, USA). See Supplementary Methods for details of the parameters used for copy number calls.

Illumina SNP analysis—We used the SNPRank Segmentation and the thresholds of log₂ ratio values for gains and losses were set at 0.08 and −0.11 respectively; the thresholds for high copy number gains and homozygous deletions were set at 0.41 and −1.1, respectively. The significance threshold for segmentation was 1×10^{-7} and a minimum number of probes per segments of 3.

Agilent CytoGenomics analysis—Copy number aberration detection was performed using the ADM-2 algorithm (Threshold=10, Fuzzy Zero=on) in Agilent CytoGenomics software v3.0.2.11 (Agilent Technologies, Santa Clara, CA).

Identification of somatic copy number aberrations

The matched pair analysis, an extended algorithm in Nexus, was used to specifically identify somatic genomic aberrations in the tumor samples. In brief, each CPE and CAS sample was compared to its corresponding BPE and BAS counterpart to identify only those events in the

tumor that were not present in the benign tissue. The two files (from tumor and normal) were combined into one result after the values in the benign sample were subtracted from that in the tumor sample (log ratios are subtracted). This procedure removed a substantial number of identified aberrations representing structural variants in the germ-line. Second, no SCNAs were called positive if absent in its respective CPE or CAS before the matched paired analysis, or if the aberration was present in either BPE or BAS from same patient, irrespective of the cell type.

Detection of TP53 mutations by direct sequencing

Six sets of primers were used to amplify DNA fragments covering exons 4 to 9 of the TP53 gene (primers available on request) for subsequent Sanger sequencing and identification of mutations. See Supplementary Methods for details of the sequencing methods. The sequence variant identified in sample 64819 CPE was confirmed with a second PCR-sequencing reaction using a new set of primers for exon 4 obtained from the IARC TP53 Database.

mtDNA mutations

To ensure accurate identification of homoplasmic tumor mtDNA mutations, patient-matched normal peripheral blood cells, microdissected prostatic carcinoma and regions of benign stroma were isolated using laser capture microdissection (LCM) from surgically resected tumors (radical prostatectomies). The entire mitochondrial genome was sequenced in prostatic cancer, prostate stroma, and blood samples from each patient first by PCR amplifying the mtDNA with 28 pairs of primers, as previously described (12). Clonally expanded mtDNA mutations were scored only when the sequence of the tissue samples differed from that of the patient-matched normal peripheral blood cells. All regions with detected mutations were re-amplified and sequenced to rule out the possibility of the mutations being produced by polymerase errors during the PCR or sequencing processes. In addition, to guard against the sample mix-up and contamination that has confounded many mtDNA mutation studies (13), we compared each patient's sequences to the revised Cambridge Reference Sequence (rCRS) to confirm they shared common polymorphisms.

Immunohistochemistry

The polyclonal antibody, HOXA9 (Sigma HPA061982), was used to determine the percentage of stromal cells staining for different intensity levels of nuclear HOXA9 in prostate cancer and benign prostate using a tissue microarray (TMA) comprised of tissue cores from radical prostatectomies. TMA slides were then scanned at $\times 40$ magnifications using the Aperio scanner. Additional details are provided in Supplementary Extended Experimental Methods.

RESULTS

Genomic Copy Number Alterations in Prostate Cancer and Cancer-Associated Stroma

To identify clonal somatic structural alterations in the genomes of prostate carcinoma cells and adjacent stroma, we used laser capture microdissection to acquire populations of cells from fresh frozen prostate tissues procured from 20 patients undergoing radical prostatectomy. We isolated cells of four spatially and histologically-defined cell phenotypes:

prostate cancer epithelium (CPE), benign prostate epithelium (BPE), prostate cancer-adjacent stroma (CAS), and benign prostate epithelium-adjacent stroma (BAS). For CAS, all captured stromal cells were within 1 mm of a cancer focus. Tumor CPE and CAS were microdissected from adenocarcinomas with Gleason pattern 3 or 4.

For BPE and BAS samples, morphologically benign regions were identified at least 10 mm distant to any cancer foci, or more commonly, from a sample in a different quadrant of the prostate with no histological evidence of carcinoma. A typical example of microdissected epithelial and stromal cells from tumor and benign prostate tissue are shown in Figure 1.

Since limited amounts of DNA were obtained from microdissected cells (11–80 nanograms), we amplified the DNA from each cell population, by whole genome amplification (WGA), and determined genome-wide DNA copy number status by microarray comparative genomic hybridization (aCGH) (14). Compared to normal reference DNA, discrete regions of copy number variation (CNVs), between 6 and 26 regions per case, were identified in all epithelial and stromal samples including those from regions with or without carcinoma (See Table S1, fourth column, CNV). Single nucleotide variants and differences in DNA sequence encompassing small and large chromosomal regions are well-documented to occur as polymorphisms in the non-diseased population with considerable normal variation between individuals (15,16). We hypothesized that a substantial number of the CNVs concordantly observed in all cell populations from a given individual represented germ-line differences between patients and the reference DNA. A CNV was clearly detected in regions with as few as 6 probes. On the basis of this observation, and in order to increase the chances of identifying any genomic copy number aberration in CAS, we maintained the default settings on the software algorithm used for initially calling CNVs (see Methods).

Following the removal of germ-line variants and applying several filtering criteria (see Methods), we evaluated prostate carcinoma cells (CPE) for evidence of SCNAs. Of the 20 tumors analyzed, we found SCNAs in 19, with multiple SCNAs in most CPE samples, ranging from 5 to 74 per tumor (mean value = 25 ± 17 SCNAs/case), for a total of 503 SCNAs across all tumors. The length of the genomic aberration in CPE ranged from 16 kb to 100 Mb, and contained between 3 to 15531 probes (Figure 2 and Tables S1 and S2). We identified several regions in CPE that occurred in more than one tumor (111 SCNAs, 10% frequency), with 17 regions altered in 20% of tumors (see Table S3 for complete list of recurrent regions). Numerous studies have identified regions of copy number loss and gain that are recurrently observed in primary prostate cancer (17–21), and the tumor genomes in our analysis harbored many of these established changes including gains at 3q, 7p, 8q and 9q; and losses at 8p, 10q, 16q, 13q, 6q, 21q, among others (Figure 2B and Table S3).

In contrast to the prostate carcinoma cells, we did not detect any SCNAs in the CAS samples in 8 of the 20 cases analyzed. However, a region of SCNA was called by the rank segmentation algorithm in 12 CAS samples, ranging from 1 to 8 CAS-SCNA per tumor, with a total of 39 regions identified across these cases (Figure 2 and Tables S1 and S4). We compared each CAS-SCNA with those identified in CPE and identified 13 regions of overlap between the CPE and CAS components of the tumor. The remaining 26 CAS-SCNAs were considered CAS-unique in contrast to 490 CPE-unique SCNAs. The sizes of

the unique CAS-SCNAs ranged from 47 kb to 2 Mb comprising 14 to 313 probes. Of these CAS-SCNAs, five were recurrent in 2 CAS samples (10% frequency) and one was observed in 3 different CAS samples (15% frequency) (Table S5). Of note, although these six regions were considered CAS-unique within a given patient, the same copy number loss occurred in benign stroma and/or benign epithelium from other individuals (Table S5, **columns P-EK**), suggesting that those aberrations may not confer a selective advantage towards a cancer phenotype.

Previous studies have demonstrated significant under-amplification bias in regions with high GC content and subtelomeric regions (14,22). To remove false-positive calls due to amplification bias, we evaluated each CPE-unique (490 SCNA), CAS-unique (26 SCNA) and CPE/CAS-overlapping (13 SCNAs) regions for GC content and chromosomal location. Of the CPE-unique calls, 7 SCNAs (1%) exhibited high GC content (>53% GC in region). In the CAS compartment, 9 SCNAs (representing 35% of all CAS SCNAs) contained a GC content higher than 53%, and for the CPE/CAS overlapping regions 1 SCNA had a CG content >53%. To quantify the number of SCNAs localized in subtelomeric regions, we employed an arbitrary length and distance from chromosome termini (<2 Mb from chromosome tip and the SCNA region smaller than 2 Mb). Ten SCNAs in CAS (38%) were located in subtelomeric regions compared to fourteen SCNAs in CPE (3%) and one SCNA in CPE/CAS-overlap (8%) (Tables S2 and S4 list regions with high GC content and subtelomeric regions in CPE and CAS respectively).

Taken together, the absence or low incidence of CAS-unique SCNAs across the 20 prostate cancer cases examined, the known false-positive calls that may arise due to amplification biases, and the fact that the 6 recurring CAS-SCNAs were not exclusively found in neoplastic cell types, suggests that a substantial number of these 26 CAS-unique SCNAs resulted from technical sources of variation. To identify robust, high-confidence somatic copy number aberrations and further remove variation due to data processing, we analyzed the aCGH data using a second independent algorithm (the Agilent CytoGenomics, ADM-2) and used an automated bioinformatics filtering approach to identify unique SCNAs in CPE and CAS (see Extended Experimental Methods). Using this strategy, we identified 339 unique CPE-SCNAs and 25 unique CAS-SCNAs, compared to 490 unique CPE-SCNAs, and 26 unique CAS-SCNAs identified using the Nexus algorithm (Tables S6 and S7 for CPE- and CAS-unique SCNAs, respectively). Across the tumors, 215 and 3 SCNAs were recurrent in CPE and CAS respectively (Tables S8 and S9).

We next ascertained the copy number status of specific genes located within the regions of SCNAs called in common by each algorithm that were unique to either CAS or CPE. In the CPE samples, a high percentage of genes, 84% and 86%, within areas of gain and losses respectively, were identified by both algorithms (Figure 3A–B and Table S10 for complete gene list). In contrast, 8% of genes located within the 3 regions of copy number loss in the CAS samples overlapped between both analyses, and no genes within the amplified regions were in common between both analyses (Figure 3C–D, and Table S10). The only recurrent aberration in CAS called in both the Nexus and Agilent analyses corresponds to a copy number loss in chromosome 7 (40 probes in region covering 0.11 Mb) identified in the CAS

samples from 3 tumors (Nexus) and 2 tumors (Agilent), respectively. Genes within this region include the homeobox A gene cluster comprising 11 HOX genes (HOXA).

To confirm the copy number loss encompassing the HOXA gene cluster identified by aCGH, we performed an independent copy number assay using droplet digital PCR for two genes contained within the deleted region of chromosome 7 (*HOXA5* and *HOXA10*). To avoid bias due to DNA amplification, we laser capture microdissected and extracted an additional 10 ng of gDNA from the four cell compartments (BAS, BPE, CAS and CPE) of two cases identified as having a copy number loss in their CAS samples by aCGH. The unamplified gDNA was used as a template for the ddPCR reaction. To calculate the copy number for each experimental gene we used *VOPPI* as a reference gene. By ddPCR, no chromosome loss involving *HOXA5* or *HOXA10* was identified in any of the four cell compartments, including CAS: each cell population exhibited the expected two copies per diploid genome (Figure 3E).

Gene Expression Associations With Stromal Copy Number Aberrations

To ascertain whether regions of copy loss in CAS identified by aCGH are associated with altered gene expression, we examined the expression of *HOXA9*, a gene located within the HOXA cluster region of copy loss in CAS identified by aCGH. We stained a tissue microarray (TMA) comprised of cores from 23 localized prostate cancers resected at radical prostatectomy with an antibody recognizing *HOXA9*. We quantitated nuclear *HOXA9* immunoreactivity in stroma within benign glands or cancer foci, stroma-adjacent to benign glands or cancer foci, and stroma distant to benign glands or cancer foci. We found no differences in *HOXA9* protein expression between different tissue compartments (2-sided 2-sample t-tests of nuclear staining intensities) (Figure S4).

We also examined a previously published dataset comparing gene expression between CAS and BAS from men with localized prostate cancer for evidence of differential transcript levels (3). Focusing on the 3 regions within the Chr2, Chr5 and Chr7 CAS-SCNAs, no transcripts were found to be significantly different between CAS and BAS ($q < 0.05$) (Figure S5).

PTEN and TP53 Status in Cancer Adjacent Stroma

Loss or mutation of the *PTEN* tumor suppressor gene occurs frequently in primary prostate carcinoma (18). Loss of heterozygosity (LOH) involving the *PTEN* locus has also been reported to occur in the benign stroma of breast carcinomas (23), and *PTEN* activity in breast fibroblasts influences the development and progression of breast cancers in model systems (24). Thus, we next sought to determine if genomic alterations in *PTEN* occurred in the stroma of prostate cancers. The 400K microarrays used in this study included 33 probes spanning the *PTEN* gene on chromosome 10q, from 10 kb upstream of the transcription start site to the end of exon 9. In accordance with previous studies of prostate cancer (2), we identified heterozygous or homozygous copy number losses involving *PTEN* in 45% of the tumor epithelial samples. Conversely, we did not detect any *PTEN* loss in CAS except in one patient (case 54371) in which a heterozygous *PTEN* deletion was observed (Table 1). In this patient, a homozygous *PTEN* deletion was also present in the corresponding CPE sample.

To validate the loss of *PTEN* in the tumor adjacent stroma, we performed fluorescence *in situ* hybridization (FISH) using sections adjacent to those used for the original microdissection for CGH arrays. Homozygous deletion of *PTEN* in tumor epithelium was readily detectable; however we did not appreciate any *PTEN* loss in the CAS compartment (Figure S1), suggesting that the array CGH results most likely represented contamination of tumor epithelial cells in the stroma sample. Although we used microdissection to enrich for specific cell types, cross-contamination can occur as glands comprising tumor cells can be cut tangentially producing a section where tumor cell characteristics are not readily discernable. Of the 39 SCNAs identified in CAS, thirteen overlap directly with SCNAs in the corresponding CPE samples. Moreover, the majority of the SCNA calls in CAS also corresponded to cases where alterations in the CPE comprised homozygous losses or high copy number gains.

Loss or mutation of the p53 tumor suppressor gene (*TP53*) also occurs in primary prostate carcinoma (25) and has been reported to be somatically mutated in the stroma of human breast cancers(26) and a murine model of prostate cancer (27). The microarrays used in this study comprised 8 probes spanning the p53 locus. Of the 20 prostate cancers analyzed, 6 CPE were found to have a single copy loss of *TP53* (Table 1). No SCNAs in the region of *TP53* were found in any CAS sample. We determined the nucleotide sequence of *TP53* (exons 4–9) in 10 cases as somatic mutation frequencies as high as 50% have been reported in cancer-associated fibroblast from other tissues (26,28). A somatic mutation in *TP53* was identified in the CPE sample of one patient (Figure S2). This mutation has been reported as a somatic mutation in 12 tumors in the IARC TP53 Mutation Database version R15 (29). No somatic *TP53* mutations were detected in the CAS from any of the 10 cases analyzed, a finding which is in agreement with previous reports using fresh frozen microdissected regions of stroma from breast and ovarian cancers (30,31).

Focused Assessments of Chromosome Regions Associated with Prior Studies Reporting Allelic Imbalance in Prostate Cancer-Adjacent Stroma

A previous report using microsatellite markers to identify chromosomal abnormalities in primary prostate cancers and cancer-adjacent stroma identified 8 regions of recurrent loss of heterozygosity (LOH) or allelic imbalance (AI) that occurred specifically in prostate cancer-associated stroma (10). We manually reviewed the copy number calls and the probe intensity plots derived from the aCGH hybridizations corresponding to each of these 8 stromal LOH/AI regions and extended the analysis to ± 250 kb from each of the designated microsatellite markers. We did not detect any change in copy number corresponding to any of the 8 reported regions of CAS-associated LOH/AI in the CAS samples compared to matched BAS or the BPE compartments (Table S11). Notably, CN losses were observed in CPE for markers that were proposed to be CAS specific (e.g. D12S1045, D14S606 and D2S434). Since we could not rule out copy neutral LOH in CAS samples for these 8 microsatellite markers, we performed PCR-based microsatellite analysis on our sample cohort and focused on two previously identified markers showing AI/LOH specifically in CAS (D2S1400 and D12S1045) (10). Microsatellite analysis for markers D2S1400 and D12S1045 did not detect LOH in any CAS, however LOH for marker D12S1045 was detected in a tumor epithelium sample from one patient (58487 CPE) and was also reflected

as a copy number loss in the CGH array (Figure S3 and Table S11). We cannot rule out copy neutral LOH in CAS samples for the other 6 stroma-associated microsatellite markers. However, the CGH data indicates that if allelic alterations are present in prostate CAS, they are not associated with copy number changes in their genome.

Mitochondrial DNA Mutations in Prostate Cancer and Prostate Stroma

Mutations in mitochondrial DNA (mtDNA) have been reported to occur in prostate carcinoma and represent a convenient analyte due to the abundance of mitochondrial DNA with multiple copies of the mitochondrial genome per cell. We next sought to determine if somatic mutations in the mitochondrial genome are present in benign prostatic stromal cells. We evaluated an independent cohort of 40 primary prostate carcinomas and isolated DNA from patient-matched normal peripheral white blood cells and microdissected stroma and cancer epithelium from surgically resected tumors (radical prostatectomies). We sequenced the entire mitochondrial genome from each sample after first amplifying the entire mitochondrial genome with 28 pairs of primers (12). Clonally expanded mtDNA mutations were scored only when the sequence of the tissue samples differed from that of the patient-matched peripheral blood cell DNA. All regions with detected mutations were re-amplified and sequenced to rule out the possibility of the mutations being produced by polymerase errors during the PCR or sequencing processes. In addition, to confirm the assignment of each sample to a particular individual, we compared each patient's sequences to the revised Cambridge Reference Sequence (rCRS) to confirm they shared common polymorphisms. We identified somatic mtDNA mutations in 24 of 40 microdissected cancer epithelium samples (60%) ranging from 1–3 mutations per patient. There were no recurrent mutations in specific nucleotides in the cancer samples among the 40 patients, although mutations in different regions of the same gene occurred including mutations in *Cytb* and the D-loop in five individuals. In contrast, we identified 1 stromal sample with one mtDNA mutation (11256A>G in *ND4*) (see Table 2). *ND4* was not mutated in the cancer epithelium samples.

Genomic Copy Number Alterations in Cultured Prostate Cancer-Associated Fibroblasts

To further address experimental findings that could be influenced by the contamination of stromal cell preparations with carcinoma cells, and to assess potential genomic events that associate with a stromal cell population clearly capable of promoting prostate tumorigenesis, we analyzed primary cultures of cancer associated fibroblasts (CAFs), and fibroblasts isolated from regions of the prostate without neoplastic cells (NAFs). All of the CAF and NAF preparations were passaged a minimum of 6–8 times *in vitro* to ensure the elimination of epithelial cells.

We first assessed DNA copy number by aCGH in two CAF-NAF pairs in which the CAF cells and NAF cells have been previously shown to be capable or incapable of promoting tumorigenesis respectively, in mouse xenograft models (4,11). Overall, we identified discrete regions of copy number differences (21 and 17 CNVs, respectively) in both CAFs and NAFs compared with the normal reference DNA sample, a finding consistent with the number of germ-line variants identified in tissue samples using the same Agilent CGH platform. However after excluding the presumed germ-line variants by matched-pair analysis with NAF DNA, and applying the filtering criteria where a CN aberration will be called positive

if the aberration is present after the matched-pair analysis, none of the CAFs showed evidence of copy number gains or losses in any chromosome (Figure 4). In order to increase the exonic coverage and examine allele ratios, three independent CAF-NAF pairs were analyzed using a single nucleotide polymorphism (SNP) array platform (Illumina Infinium HumanOmniExpressExome BeadChip) which comprises more than 240,000 functional exonic markers, and more than 700,000 genome-wide markers. No unique genomic aberrations were identified in CAF compared to NAF samples. Additionally, no copy-neutral LOH associated with CAF cells were identified (Figure 4).

DISCUSSION

In this study we sought to determine if clonal genomic aberrations are present in benign stromal cells located adjacent to cancer cells in the microenvironment of the prostate gland. We used array CGH, DNA sequencing, and microsatellite analyses to evaluate the genomic DNA of microdissected prostate cancer-associated stroma (CAS) isolated from fresh frozen tissue, and from cultured prostate cancer-associated fibroblasts (CAF). Although somatic genomic aberrations were readily identified in prostate cancer cells, we found no convincing evidence of clonal somatic copy number alterations in the stromal compartment. Targeted mutational analysis demonstrated no evidence of TP53 mutation in CAS. In addition to DNA copy number data, our analysis of CAF DNA on SNP arrays revealed no copy neutral LOH regions (areas of genomic LOH without loss of DNA). Moreover, although most cancerous epithelial cells harbored mutations in the mitochondrial genome, in only one case was a mitochondrial mutation identified in the stroma. Taken together, these results demonstrate that the genomes of stromal cells associated with prostate cancer are for generally stable and indistinguishable from the benign stroma and benign epithelial cells from the same patient.

In contrast to the absence or very low incidence of genomic alterations in cancer-associated stroma cells, the cancer epithelial cells (CPE) contained widespread gains and losses across their genomes including several aberrations previously reported to occur in prostate cancers (18). Examples of recurrent aberrations included loss of PTEN and TP53 and gain of MYC, loss of chromosome 8p, and gain of chromosome 8q. The identification of these genomic aberrations in our study demonstrated the feasibility of the approach using minute quantities of DNA obtained by laser capture microdissection, amplified by WGA and hybridized into aCGH for the identification of SCNAs in specifically defined cell populations.

Although the majority of the CAS samples showed a diploid genome, a few regions of copy number gain and loss were identified by two independent algorithms. However the agreement between both analyses was minimal. Several of these SCNAs overlapped with alterations found in patient-matched cancer cells which could be due to contamination of the isolated stroma compartment by infiltrating tumor cells (32). Of the CAS-unique SCNAs, a subset were located in sub-telomeric regions and/or were high in GC content, suggesting that a high percentage of genomic aberrations found in CAS may be arising from amplification artifacts. LCM-WGA DNA samples have two major technical challenges: first, the quality and quantity of DNA that can be obtained is often limited and can result in low-confidence CGH analysis calls (33). Second, it is known that WGA exhibits amplification

biases; many amplification artifacts generated by WGA are reproducible, and may correlate with proximity to chromosome ends and GC content (14,22,34,35). After accounting for possible artifacts, only 3 regions with SCNA loss were consistently called using the two analysis algorithms. Notably, these regions were located in gene clusters, one within the protocadherin beta cluster (PCDHB) and the other within the HOXA cluster, that comprise duplicated genes with repeats of highly homologous nucleotide sequences. Using a digital droplet PCR method on unamplified DNA we were unable to reproduce the aCGH finding involving the HOXA region suggesting that the copy number loss stems from a technical artifact of the methodology that included DNA amplification.

Our results are in agreement with previous reports demonstrating that genomic alterations do not typically occur in cancer associated stromal cells. A study of breast carcinomas assessing purified cell types represented in normal breast tissue and breast carcinomas identified a substantial number of mRNA changes in each distinct cell type, but genomic alterations determined by CGH and SNP assays were only detected in cancer epithelial cells (36). SNP arrays and microsatellite markers (MSM) were used to analyze the genomes of microdissected frozen CAS from 25 and 10 ovarian and breast cancer patients, respectively, and 6 primary cultures of breast CAFs, and demonstrated no genomic alterations in any of the stroma samples excepting a chromosomal 22 loss in one ovarian sample (37). aCGH and targeted TP53 sequencing were used to assess genomic aberrations in 25 fresh cultured breast CAF samples and found no copy number changes in their genomes nor were TP53 mutations detected except in one case (31). A study involving 7 primary cultures of pancreatic CAF and a tissue microarray containing 117 pancreatic adenocarcinoma cases did not identify SCNAs or immunohistochemical evidence of TP53 mutations (38). An analysis of 40 colorectal carcinomas determined that stromal cells do not share KRAS mutations that are present in the corresponding carcinomas. No somatic genomic aberrations were identified in SNP array-based analyses of stromal cells derived from 57 FFPE cervical carcinomas or in a study that examined 99 fresh frozen breast cancers (39).

Our findings and those described above contrast with several studies reporting high frequencies of genomic aberrations (e.g. LOH, and point mutations of tumor suppressor genes) in benign stromal cells derived from breast, ovarian, pancreas and prostate carcinomas ((10) and reviewed in (5)(40)). The commonality between these studies was the use of archival FFPE tissue. It has been suggested that the detection of chromosomal aberrations and mutations may be influenced by FFPE-induced DNA artifacts that produce false positive results due to the low yields, DNA cross-links and fragmented/degraded DNA (5,37). A study using microsatellite markers to define genomic aberrations in FFPE archived samples of prostate cancer identified 8 recurrent regions of LOH in cancer-associated stroma. We evaluated two of the microsatellite markers that defined regions of LOH and found no evidence of LOH in our CAS samples, though one CPE sample exhibited allelic imbalance across the D12S1045 microsatellite.

It is possible that genomic alterations may occur in individual benign cells, such as cancer-associated stroma cells, as it has been observed that individual neurons from normal human brain tissue can exhibit structural chromosomal alterations (41,42). However, although single cells or minor subpopulations of benign-appearing stroma cells may harbor DNA

aberrations, the fact that we did not observe recurrent clonal SCNAs suggest that those aberrations, if they occur, do not present a selective advantage for cell survival or growth. The fact that proliferation rates in CAS are extremely low, in contrast to carcinoma cells, also suggests that there is very limited clonal expansion of stromal cells carrying somatic genetic changes. Through a whole genome sequencing analysis involving multi-region sequencing of 3 prostate cancer cases (multiple benign and cancer foci per case), somatic mutations in morphologically normal prostate epithelium was observed which suggested that clonal expansions or fields of cells may provide a background against which prostate cancer develops (43). In our study, we did not observe a substantial number of aberrations in BPE that would suggest clonal expansion of benign cells. However, our CGH-based analyses do not have the resolution of deep sequencing to detect very minor clonal populations of cells within a complex tumor microenvironment.

It is well recognized that fibroblasts derived from the microenvironment regions adjacent to cancer cells exhibit substantial changes in gene expression. Our study, documenting that clonal somatic genomic alterations in the CAS cells are rare, if not absent, suggests that the gene expression changes observed in CAFs and CAS represent responses to extrinsic signals derived from tumor cells or infiltrating inflammatory cells that may comprise a spectrum of growth factors, cytokines, and matricellular proteins. Alterations in gene expression may also be mediated via epigenetic changes. Several studies including those involving breast and prostate cancers have identified distinct differential methylation profiles when comparing stromal cells derived from microenvironments of benign tissues compared to regions with cancer (44–46). Understanding the mechanisms responsible for such epigenetic modification is an active area of investigation that may have relevance in the context of therapy in view of the potential for reversing epigenetic states. In conclusion, our data provide evidence that recurrent SCNAs, copy neutral LOH or TP53 mutations are absent or very rarely occur in the cancer-associated stroma from prostate carcinomas. Thus, molecular mechanisms other than chromosomal aberrations underlie the pivotal role of CAS in the initiation and progression of prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Implications

The gene expression changes observed in prostate cancer-adjacent stroma and the attendant contribution of the stroma to the development and progression of prostate cancer are not due to frequent or recurrent genomic alterations in the tumor microenvironment.

Figure 1. Laser capture microdissection of epithelial and stromal cells from fresh frozen prostate tissue

(A) Image of 7 μ m prostate tissue section on PEP membrane slide. (B) Image of tumor region of interest. (C–D). An area cancer adjacent stroma (*) before (C) and after (D) LCM. E–F, an epithelial area (+) from the same tumor region before (E) and after (F) LCM. (G–H), Patient-matched benign epithelium (G) and stromal (H) areas microdissected from regions away from the tumor or from other tissue blocks (preferentially). Delineated areas in black and marked (+) are the benign epithelium and stromal adjacent to benign epithelium areas, immediately after LCM but before lifting the cap from the whole tissue section.

Figure 2. Genomic copy number aberrations in prostate cancer epithelium and cancer-associated stroma

(A) Number of somatic copy number aberrations (SCNAs) found within each case in both CPE and CAS. Red, SCNAs identified as unique to CPE. Blue, SCNAs identified as unique to CAS, green, SCNAs identified as overlapping between CAS and CPE. Note the low number of CAS unique SCNAs as compared to SCNAs unique to CPE. (B–C) Frequency plots of DNA copy number alterations in the genome of prostate cancer. The y-axis indicates the percentage of the population in the selected samples (B) CPE matched paired samples (n = 20) and (C) CAS-matched paired samples (n = 20) having a copy number aberration event at a specific point along the genome. Blue indicates copy number gain events (above the 0% baseline) and red, copy number loss events (below the 0% baseline). The X-axis represents the position of the genome on each chromosome. Each chromosome was designated by its corresponding number and the divisions between individual chromosomes are shown by vertical lines. Note the high number of genomic aberrations and high frequency of SCNAs found in the tumor epithelium, in great contrast to the tumor stromal which exhibits a very low number of genomic aberrations and a low frequency of SCNAs across the whole genome.

Figure 3. Comparison of genes found in aberrations unique to CPE or CAS using different copy number calling algorithms

Venn diagram comparing genes identified by Nexus and Agilent CytoGenomics algorithms for the unique CPE-SCNAs gains (**A**) and losses (**B**). Venn diagram comparing genes identified by Nexus and Agilent algorithms for the unique CAS-SCNAs gains (**C**) and losses (**D**). The majority of genes identified in the CPE samples by Nexus are also found in the Agilent analysis (84% gains and 86% losses). In contrast, only 8% of genes within copy number losses in the CAS samples overlapped between both analyses. (**E**) ddPCR

measurements of HOXA5 and HOXA10 copy numbers from unamplified DNA from the 4 different cell compartments from two patients identified by aCGH to have copy number losses in the HOXA gene cluster (cases 10-011 and 00-081). No copy losses of HOXA5 or HOXA10 were identified in any cell compartment (BAS, BPE, CAS and CPE): each had 2 copies per diploid genome.

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Figure 4. Absence of DNA copy number alterations in primary cancer-associated fibroblast (CAF) cell cultures

Whole genome plots are shown for CAF1/NAF1-matched pair (A) and CAF2/NAF2-matched pair (B), and a representative CGH plot from cancer epithelium (C). Several alterations are detected in the epithelial tumors (arrows) with the absence of SCNAs in the cancer-associated fibroblast (CAF1 and CAF2). (D) aCGH/SNP array platform and number of copy number aberrations (CNAs) and somatic CNAs (SCNAs, CAF-NAF) identified in each CAF sample. Note the absence of SCNAs in the CAF samples after subtracting the CNAs present in the matched (NAF) normal pair.

Table 1
PTEN and TP53 genomic alteration in prostate tumor epithelium and tumor stroma

Case	CPE PTEN CNA	CAS PTEN CNA	CPE TP35 CNA	CAS TP53 CNA	CPE TP53 mutations	CAS TP53 mutations
01-127	loss	no	loss	no	-	-
06-066	loss	no	loss	no	-	-
10-011	loss	no	loss	no	-	-
00-081	loss	no	loss	no	-	-
55840	loss	no	no	no	no	no
64589	loss	no	no	no	no	no
00-084	loss	no	no	no	-	-
10-004	loss	no	no	no	-	-
54371	loss	loss	no	no	no	no
58487	no	no	loss	no	no	no
64819	no	no	loss	no	T125M	no
58754	no	no	no	no		no
64975	no	no	no	no		no
69654	no	no	no	no		no
10-008	no	no	no	no	-	-
10-016	no	no	no	no	-	-
10-017	no	no	no	no	-	-
60799	no	no	no	no	no	no
56465	no	no	no	no	no	no
10-009	no	no	no	no	-	-

Table 2

Clonal mtDNA mutations identified in prostate cancer epithelium and benign stroma

Patient ID	Gleason Score ^a	Gene ^b	DNA ^c	Protein ^d	PCE (%) ^e	Stroma (%) ^f	Blood (%) ^g
23300B	3+3	ND5	13913T>C	L526P	50	N.D.	N.D.
22871B	3+3	ATPase6	9038T>C	M171T	50	N.D.	N.D.
23388H	3+3	None	-	-	-	-	-
23529W	3+3	16S	rRNA	2107G>A	100	N.D.	N.D.
23481P	3+3	None	-	-	-	-	-
19575Y	3+3	None	-	-	-	-	-
22951A	3+3	None	-	-	-	-	-
22949P	3+3	tRNAE	E	14724G>A	50%	N.D.	N.D.
23127O	3+3	None	-	-	-	-	-
22904L	3+3	ND1	3982G>A	A226T	80%	N.D.	N.D.
		Cytb	15345T>C	L200S	70%	N.D.	N.D.
22880H	3+3	COXIII	9942G>A	D246N	50%	N.D.	N.D.
23024K	3+3	None	-	-	-	-	-
23270S	3+3	None	-	-	-	-	-
22905A	3+4	None	-	-	-	-	-
23002C	3+4	None	-	-	-	-	-
19416E	3+4	Cytb	14774insC	frameshift	100%	N.D.	N.D.
22860F	3+4	D-loop	523delAC	-	100%	N.D.	N.D.
		12S rRNA	1282G>A	-	80%	N.D.	N.D.
		ND3	10320A>G	188V	100%	N.D.	N.D.

Patient ID	Gleason Score ^a	Gene ^b	DNA ^c	Protein ^d	PCE (%) ^e	Stroma (%) ^f	Blood (%) ^g
23027B	3+4	COXI	6131A>G	-	60%	N.D.	N.D.
		COXI	6910C>T A336V	-	80%	N.D.	N.D.
22870S	3+4	D-loop	16171G>A		60%	N.D.	N.D.
22975M	3+4	None	-	-	-	-	-
23201A	3+4	ND3	10264T>C	I69T	70%	N.D.	N.D.
23028W	3+4	None	-	-	-	-	-
23266N	3+4	None	-	-	-	-	-
23312L	3+4	None	-	-	-	-	-
23159R	3+4	None	-	-	-	-	-
22896V	3+4	None	-	-	-	-	-
23204T	3+4	None	-	-	-	-	-
23378W	3+4	ND1	3643G>A	V113I	60	N.D.	N.D.
23390H	3+4	None	-	-	-	-	-
23569O	3+4	ND3	10228T>C	L57S	100	N.D.	N.D.
23036D	4+3	ND5	13525G>A	E397K	100	N.D.	60
23171D	4+3	D-loop	313del CCCCCTTCT	-	50	N.D.	50
		ND2	4752T>C	S95P	100	N.D.	50
22962M	4+3	D-loop	309delC		50	N.D.	N.D.
22959H	4+3	None	-	-	-	-	-
23253G	4+3	Cytb	15750T>C	L335P	100	N.D.	N.D.
22927H	4+3	D-loop	251G>A		70	N.D.	N.D.

Patient ID	Gleason Score ^a	Gene ^b	DNA ^c	Protein ^d	PCE (%) ^e	Stroma (%) ^f	Blood (%) ^g
		Cytb	14846G>A	G34S	100	N.D.	N.D.
22888L	4+3	None	-	-	-	-	-
23168H	4+3	ND5	12473T>C	I46T	80%	N.D.	N.D.
23183S	4+5	ND5	13480G>A	G382stop	80%	N.D.	N.D.
		Cytb	14798T>C	F18L	50%	N.D.	N.D.
23570B	3+3	ND4	11256A>G	Y166C	N.D.	60	N.D.
23461T	4+3	None	-	-	-	-	-

^aTumors were graded based on the Gleason Grading System, with the first number indicating the grade of the majority (>50%) of the tumor (on a scale from 1–5), and the second number signifying the grade of the minority (<50%, but >5%) of the tumor.

^bThe region or gene in the mitochondrial genome where the mutation was detected by Sanger sequencing

^cThe mutation is indicated by the base position in mtDNA, followed by the type of change. A T to C substitution at position 1000 would be described as 1000T>C, while a deletion of a G at position 500 would be 500delG.

^dAmino acid change resulting from the mutation, indicated by the original amino acid followed by the position of the residue, and then the resulting amino acid.

^ePrevalence of mutation in LCM cancerous prostate tissue as a percentage, based on Sanger sequencing chromatogram reads. N.D. indicates that the mutation was not detectable via Sanger sequencing.

^fPrevalence of mutation in LCM benign prostate stroma as a percentage, based on Sanger sequencing chromatogram reads.

^gPrevalence of mutation in blood DNA as a percentage, based on Sanger sequencing chromatogram reads.