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Identification of candidate prostate cancer genes through comparative expression-profiling of seminal vesicle

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Abstract

Background—Prostate cancer is the most frequently diagnosed cancer among men in the United States. In contrast, cancer of the seminal vesicle is exceedingly rare, despite that the prostate and seminal vesicle share similar histology, secretory function, androgen dependency, blood supply, and (in part) embryonic origin. We hypothesized that gene-expression differences between prostate and seminal vesicle might inform mechanisms underlying the higher incidence of prostate cancer.

Methods—Whole-genome DNA microarrays were used to profile gene expression of 11 normal prostate and 7 seminal vesicle specimens (including 6 matched pairs) obtained from radical prostatectomy. Supervised analysis was used to identify genes differentially expressed between normal prostate and seminal vesicle, and this list was then cross-referenced to genes differentially expressed between normal and cancerous prostate. Expression patterns of selected genes were confirmed by immunohistochemistry using a tissue microarray.

Results—We identified 32 genes that displayed a highly statistically-significant expression pattern with highest levels in seminal vesicle, lower levels in normal prostate, and lowest levels in prostate cancer. Among these genes was the known candidate prostate tumor suppressor *GSTP1* (involved in xenobiotic detoxification). The expression pattern of *GSTP1* and four other genes, *ABCG2* (xenobiotic transport), *CRABP2* (retinoic acid signaling), *GATA3* (lineage-specific transcription) and *SLPI* (immune response), was confirmed by immunohistochemistry.

Conclusions—Our findings identify candidate prostate cancer genes whose reduced expression in prostate (compared to seminal vesicle) may be permissive to prostate cancer initiation. Such genes and their pathways may inform mechanisms of prostate carcinogenesis, and suggest new opportunities for prostate cancer prevention.

Keywords

prostate cancer; seminal vesicle; expression profiling; microarray

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Introduction

Anatomically, the glandular prostate can be divided into the peripheral zone (PZ), central zone (CZ) and transitional zone (TZ), arising embryologically from the urogenital sinus (PZ, TZ) and Wolffian (mesonephric) duct (CZ) [1]. Approximately 60% of prostate cancers arise in the PZ, while another ~20% arise in each of the CZ and TZ [2]. The normal prostate functions to secrete components of the seminal fluid, including acid phosphatase, proteases, polyamines, citric acid and zinc [3]. Histologically, prostate ducts are lined by two layers of epithelium, a columnar secretory layer surrounded by a cuboidal basal layer, embedded in a fibromuscular stroma. Both epithelial growth and function of the prostate are androgen dependent [4].

In close anatomic proximity to the prostate is another (paired) accessory sex gland, the seminal vesicle. The seminal vesicle also secretes components of the seminal fluid, including fructose, semen coagulators, sperm motility enhancers, antioxidants and immune response modulators [5]. In addition to its seminal secretory function, the seminal vesicle shares other features with the prostate, including an embryonic origin from the Wolffian duct (like prostate CZ), a bilayered tubuloalveolar histology, androgen dependent growth and function, and a common blood supply (and therefore carcinogen exposure) from branches of the internal iliac artery [5,6]. While seminal vesicle histology resembles that of the prostate, particularly CZ [1], differences are noted including that the basal cell layer is discontinuous and neuroendocrine cells are lacking in seminal vesicle epithelium [7].

Despite the overall similarities, the incidence of cancer of the respective organs is strikingly different. While in 2007 an estimated 219,000 new cases of prostate cancer will have been diagnosed in the United States alone [8], only about 50 cases of primary adenocarcinoma of the seminal vesicle have ever been documented in the medical literature [9]. Though the volume of the paired seminal vesicle is less than that of the prostate, this alone cannot account for the more than 100,000-fold difference in cancer incidence between the two organs. Understanding the basis of the disparity in cancer incidence may provide new insights into the development of prostate cancer, and may point towards new strategies for prevention [10].

We hypothesize that the tissue-specific differences in cancer incidence might stem from differences in expressed genes, discoverable by gene expression profiling. Specifically, we propose that certain genes expressed at higher levels in seminal vesicle might be protective, while at lower levels in prostate would be permissive, to cancer initiation. Here, we carry out whole-genome microarray-based expression profiling to discover such genes.

Materials and Methods

Profiled specimens

Frozen tissue from prostate and seminal vesicle specimens was obtained from radical prostatectomy specimens with IRB approval and patient informed consent. The absence of cancer or necrosis was confirmed by H&E-stained frozen section. Tissues were homogenized and total RNA isolated by Trizol (Invitrogen, Carlsbad, CA) extraction, and RNA quality confirmed by rRNA integrity using gel electrophoresis.

Expression profiling

cDNA microarrays were obtained from the Stanford Functional Genomics Facility (SFGF) and contained 39,632 non-redundant cDNA clones representing 14,613 named genes, 4,925 additional UniGene clusters [11], and 3,068 expressed sequence tags (ESTs) not mapping to UniGene clusters. Expression profiling was performed as previously described [12]. Briefly,

50µg total RNA from each sample and 50µg “universal” reference RNA (derived from 11 different established human cell lines) were differentially labeled with Cy5 and Cy3, respectively, and co-hybridized to cDNA microarrays. Following overnight hybridization and washing, arrays were imaged using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). Fluorescence ratios were extracted using SpotReader software (Niles Scientific, Portola Valley, CA), and the data uploaded into the Stanford Microarray Database (SMD) [13] for storage, retrieval and analysis. The complete microarray dataset is available at SMD and at GEO (GSE000).

Microarray data analysis

Background-subtracted fluorescence ratios were normalized for each array (i.e. global normalization), and then each gene normalized to the average expression of that gene across all samples (i.e. mean-centered). For subsequent analysis, we used only the 971 cDNAs on the microarray whose expression was both well measured and highly variable among samples. Well-measured genes were defined as those with signal intensity/background >1.5 for either the Cy5-labeled test sample or the Cy3-labeled reference, in at least 75% of samples. Variably expressed genes were those with expression at least 3-fold higher or lower from the sample average, in at least two samples. Genes differentially expressed between seminal vesicle and normal prostate were identified with the two-class Significance Analysis of Microarrays (SAM) method [14], which uses a modified t-statistic and provides a false discovery rate (FDR) estimate by comparison to randomly permuted data. SAM analysis was also used to identify genes differentially expressed between normal prostate and prostate cancer, using publicly-available cDNA microarray data from our previously published study [12]. Cross-tissue comparisons were also made using previously published expression profiles [15] for normal seminal vesicle, prostate, breast, colon and lung.

Tissue microarray construction

A tissue microarray (TMA) was constructed from formalin-fixed paraffin embedded blocks comprising 15 matched specimen sets of seminal vesicle, normal prostate and prostate cancer obtained from radical prostatectomy (including the six matched cases profiled for gene expression, plus nine additional cases), together with 7 matched sets of seminal vesicle and normal prostate from radical cystectomy cases where prostate cancer was not present. Each specimen was represented by a single 1.5 mm core.

Immunohistochemistry

A 4 µm section was cut from the TMA block, deparaffinized in Citrisolv (Fisher Scientific, Hampton, NH), and hydrated in a graded series of alcohol solutions. Heat-induced antigen retrieval was performed by microwave pretreatment in citrate (1mM, pH 6.0) for 15min before staining. Endogenous peroxidase was blocked by preincubation with 1% hydrogen peroxide in phosphate-buffered saline. Primary antibodies were used as follows: anti-ABCG2 mouse monoclonal antibody (ab3380 Novus Biologicals, Littleton, CO) at 1:100 dilution incubated 1hr at room temp; anti-CRABP2 rabbit polyclonal antibody [16] at 1:500 for 16hrs at 4°C; anti-GATA3 mouse monoclonal antibody (sc-268, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:10 for 16hrs at 4°C; anti-SLPI rabbit polyclonal antibody (ALX-210-376, Alexis Biochemicals, San Diego, CA) at 1:50 for 30min at RT; anti-GSTP1 rabbit polyclonal antibody (AB8902, Chemicon/Millipore, Billerica, MA) at 1:500 16hrs at 4°C. Chromogenic detection was carried out using the appropriate peroxidase-conjugated secondary antibody and DAB reagents provided with the Envision detection kit (Dako). Staining intensity was scored as absent (-), weak (+), or strong (++)

Results

To explore gene-expression patterns in normal prostate and seminal vesicle, we used whole-genome cDNA microarrays to profile a set of 11 normal prostate and 7 seminal vesicle specimens, including 6 matched pairs. Unsupervised hierarchical clustering of expression profiles distinguished two main groups, one containing the prostate and the other the seminal vesicle specimens (data not shown). To directly identify genes expressed at higher levels in seminal vesicle, we carried out a two-class Significance Analysis of Microarrays (SAM) analysis. In total, we identified 215 cDNAs with significantly higher expression levels in seminal vesicle, using a stringent false discovery rate cutoff ($FDR=0.045\%$) (Fig. 1A).

Based on the rarity of seminal vesicle compared to prostate cancer, we had hypothesized that genes protective against cancer might be embedded within this set of seminal vesicle tissue-specific expressed genes. An additional characteristic of such genes might be their further decreased expression in prostate cancer compared to normal prostate. That is, further decreased expression in a subpopulation of precancerous prostate epithelial cells would further favor prostate cancer initiation (and the low expression levels would be retained in the outgrowing prostate tumor). Therefore, to enrich for such candidate cancer genes, we cross-referenced the list of 215 cDNAs exhibiting higher-level expression in seminal vesicle compared to normal prostate with a SAM-derived list of 1,235 cDNAs ($FDR=0.018\%$) exhibiting significantly lower-level expression in prostate cancer compared to normal prostate (Fig. 1A), as determined from our previously published prostate cancer microarray dataset [12]. In total, the intersection of these two SAM lists comprised 38 cDNAs representing 32 different genes, each displaying highest levels of expression in seminal vesicle, lower levels in normal prostate, and lowest levels in prostate cancer (Fig. 1A, 1B and Table 1). These genes were also generally expressed at lower levels in normal breast, colon and lung, other epithelial tissues where cancer commonly develops (Fig. 3).

An additional feature of candidate tumor suppressor genes is that their expression in cancer can be silenced by promoter DNA hypermethylation [17]. We had previously identified 271 genes whose expression was upregulated in at least one of four cultured prostate cancer cell lines assayed following treatment with 5-Aza-2'-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase [18]. Such genes are enriched for those transcriptionally silenced by promoter hypermethylation in prostate cancer. It is therefore notable that 8 of the 32 candidate prostate cancer genes identified above were among the genes upregulated in prostate cancer cells upon treatment with 5-aza-dC, a highly statistically significant enrichment ($P<0.001$, hypergeometric test) (genes indicated by asterisk in Table 1).

To verify and extend our microarray findings, we sought to assess corresponding protein expression of these genes by immunohistochemistry using a tissue microarray (TMA) containing matched sets of seminal vesicle, normal prostate and prostate cancer, from the original cases profiled for gene expression as well as additional cases. We characterized protein expression for 5 of the 32 genes for which antibodies functioning on paraffin tissue were available: ABCG2, CRABP2, GATA3, SLPI and GSTP1 (Fig. 2A). Immunostaining of ABCG2 and CRABP2 was predominantly cytoplasmic, while GATA3 staining was nuclear, and SLPI and GSTP1 stained both nuclei and cytoplasm. Consistent with the microarray findings, all five proteins exhibited a statistically significant expression gradient with highest levels in the epithelia of seminal vesicle, intermediate levels in normal prostate, and lowest levels in prostate cancer ($P\leq 0.01$; χ^2 -test) (Fig. 2B).

Discussion

The prostate and seminal vesicle share similar histology, secretory function, androgen dependency, blood supply, and (in part) embryonic origin. Despite these commonalities, cancer of the prostate is very common while cancer of the seminal vesicle is exceedingly rare. We hypothesized that the difference in cancer incidence might reflect differences in expressed genes. In particular, genes expressed at higher level in seminal vesicle might protect against cancer, while those same genes when expressed at lower levels in prostate would provide more permissive conditions for cancer initiation. By expression profiling, we identified 32 genes exhibiting highest expression levels in seminal vesicle, lower levels in normal prostate, and lowest levels in prostate cancer. For a subset of these genes, we then confirmed this expression pattern across tissues for the corresponding proteins by immunohistochemistry.

Several lines of evidence suggest that the set of 32 genes is likely enriched for prostate cancer genes. First, the genes are significantly enriched for those upregulated by 5-aza-dC treatment of prostate cancer cells, consistent with promoter hypermethylation, a feature of tumor suppressor genes. Second, among the genes is a well-studied candidate tumor suppressor, *GSTP1*. Hypermethylation of *GSTP1* is among the earliest molecular alterations documented in prostate tumorigenesis [19]. It has been proposed that GSTP1 functions as a “caretaker”, protecting against oxidative damage to DNA (e.g. from dietary carcinogens) [20]. Loss of GSTP1 would therefore be permissive to cancer initiation, and indeed studies of cultured prostate cells have established that GSTP1 protects against carcinogen-associated DNA damage [21]. Here, we find that GSTP1 is expressed at highest levels in seminal vesicle, which might in part explain the rare occurrence of cancer in that organ.

Additional evidence supporting an enrichment of tumor suppressors among the 32 genes is that many of their known functions are consistent with tumor suppression. For example, ABCG2 (ATP-binding cassette, sub-family G, member 2) functions as a xenobiotic transporter [22], and like GSTP1 may protect against dietary carcinogens. TGFBR3 (transforming growth factor, beta receptor III) is a co-receptor mediating growth inhibitory TGF- β signaling, and its decreased expression has recently been linked to prostate tumorigenesis [23,24]. GATA3 (GATA binding protein 3) is a transcriptional regulator that mediates cell lineage/differentiation-specific transcriptional programs, and has been found mutated, implying a tumor suppressive role, in some estrogen receptor-positive breast cancers [25].

CRABP2 (cellular retinoic acid binding protein 2), previously identified as down-regulated in prostate cancer [26], mediates the activities of retinoic acid in cell growth, differentiation, apoptosis, and anti-carcinogenesis [27]. *GPRC5B* (G protein-coupled receptor, family C, group 5, member B), another of the 32 genes, has also been characterized as retinoic acid inducible gene 2 (*RAIG2*) [28], providing another connection to retinoic acid signaling. SLPI (Secretory leukocyte peptidase inhibitor) has anti-inflammatory, as well as pathogen defense and tissue repair activities [29]. We can speculate that decreased expression of SLPI may be permissive to inflammation, a proposed etiologic factor for prostate carcinogenesis [30].

Of course the higher incidence of prostate cancer, compared to cancer of the seminal vesicle, might result from factors other than differentially expressed genes. For example, chronic prostatitis is common and inflammation can be found on most radical prostatectomy specimens (though typically without an identified bacterial or viral source) [30], while inflammation of the seminal vesicle is not frequently described. Nevertheless, there likely exists interplay between gene expression and such tissue-specific environments. For

example, the differential expression of genes like *SLPI* may contribute to the tissue-specific differences in inflammation. It is likely therefore that gene expression differences contribute in large part to the disparate rates of carcinogenesis.

Prostate cancer has a relatively strong hereditary component. Linkage analysis has identified at least ten probable loci harboring prostate cancer susceptibility genes [31]. It is noteworthy that three of the 32 candidate prostate cancer genes identified in our study reside within such loci, including *CRABP2* at 1q23.1 (*CAPB* susceptibility locus), *C8orf13* at 8p23.1, and *SLPI* at 20q13.2 (*HPC20* locus). These three genes should therefore also be considered candidates for the resident hereditary susceptibility genes, where specific allelic variants might modify hereditary prostate cancer risk.

It might also be that genes expressed at higher levels in normal prostate compared to seminal vesicle promote the development of prostate cancer. A similar but “reverse” analysis identified 14 genes (Table 2) with significantly elevated expression both in normal prostate compared to seminal vesicle, and in prostate cancer compared to normal prostate, including *ENTPD5*, *SIM2*, *TARP*, previously linked to prostate tumorigenesis [32–34]. However, since cancer develops commonly in many different epithelial tissues, parsimony would support the relative importance of tissue-specific protective genes expressed at higher levels in seminal vesicle.

In summary, our microarray analysis has identified a set of candidate prostate cancer genes whose decreased expression in prostate compared to seminal vesicle might be permissive to prostate cancer initiation. Further studies are needed to establish a mechanistic connection to prostate carcinogenesis, through modifying the expression of these genes in cultured prostate cells, or in animal models, and assessing the impact on carcinogenesis. Nonetheless, our findings identify new potential links to prostate tumorigenesis, and highlight promising avenues for prostate cancer prevention [35,36]. In particular they underscore the potential roles of antioxidants, anti-inflammatory agents, retinoids, and possibly new strategies to modify TGF- β signaling. More generally, our findings underscore the potential for leveraging insights from the seminal vesicle to inform and possibly in the future reduce the risk of prostate cancer.

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Figure 1. Comparative profiling to identify candidate prostate cancer genes
(A) Flowchart illustration of strategy used to identify tumor suppressor candidates, as the intersection of genes expressed significantly more highly in seminal vesicle vs. normal prostate, and in normal prostate vs. prostate cancer. As depicted in the Venn diagram, the intersection comprises 38 cDNAs representing 32 different genes. (B) *Left*, Heatmap representation of the 32 candidate prostate cancer genes (38 cDNAs), ranked by SAM score in the seminal vesicle (SV) vs. normal prostate (PN) comparison. Red and green intensity indicates increased and decreased relative expression, respectively (scale indicated); gray indicates poorly measured data. *Right*, heatmap representation of average expression levels in normal prostate (PN) compared to prostate cancer (PC), scale indicated.

Figure 2. Immunostain patterns of candidate prostate cancer genes

(A) Representative immunostains of paired seminal vesicle, normal prostate and prostate cancer, for ABCG2, CRABP2, GATA3, SLPI and GSTP1. Chromogenic detection (brown precipitate), counterstained with hematoxylin; 400X original magnification. Note, in the top right panel normal prostate (N) and prostate cancer (C) are present in the same field (delineated by dashed line), highlighting the difference in ABCG2 staining intensity. **(B)** Bar graphs depict the distribution of staining intensities in seminal vesicle, normal prostate and prostate cancer specimens. Staining was scored as negative (-), weak (+) or strong (++). P-value for χ^2 test shown.

Figure 3. Expression of candidate prostate cancer genes across other normal tissues
Heatmap representation of the 32 candidate prostate cancer genes, ordered alphabetically, in normal seminal vesicle, prostate, breast, colon and lung. Expression profiling data are from [15]. Red and green intensity indicates increased and decreased relative expression, respectively (scale indicated); gray indicates poorly measured data.

Table 1
Candidate prostate cancer genes (decreased in prostate vs. seminal vesicle)

Gene symbol	Gene name	Cytoband	Biologic process	SV/PN [#]
ABCC3	ATP-binding cassette, sub-family C, member 3	17q21.33	Membrane transport	6.2
ABCG2*	ATP-binding cassette, sub-family G, member 2	4q22.1	Membrane transport	20.3
C8orf13	Chromosome 8 open reading frame 13	8p23.1 [#]	Unknown	4.4
CAPN6	Calpain 6	Xq22.3	Protease activity	5.3
CLU*	Clusterin	8p21.1	Possible role in apoptosis	6.0
CRABP2	Cellular retinoic acid binding protein 2	1q23.1 [#]	Retinoic acid signaling	5.1
CXCL6	Chemokine (C-X-C motif) ligand 6	4q13.3	Chemotaxis	3.6
DNAJC15*	DnaJ homolog, subfamily C, member 15	13q14.1	Unknown	10.7
EST	Expressed sequence tag (AA703625)	12q12	Unknown	3.5
EST	Expressed sequence tag (N90470)	4p15.32	Unknown	3.4
FADS2	Fatty acid desaturase 2	11q12.12	Fatty acid metabolism	4.6
FAM46A	Family with sequence similarity 46, member A	6q14.1	Unknown	4.3
FLJ26998	cDNA FLJ26998	10p13	Unknown	8.6
GATA3	GATA binding protein 3	10p14	Lineage-specific transcription	8.3
GLIS3	GLIS family zinc finger 3	9p24.2	Transcriptional regulation	8.1
GOLSYN	Golgi-localized protein	8q23.2	Vesicular transport	4.8
GPRC5B	G protein-coupled receptor, family C, group 5, member B	16p12.3	Unknown	4.2
GSTM2*	Glutathione S-transferase M2	1p13.3	Xenobiotic metabolism	3.3
GSTP1	Glutathione S-transferase pi	11q13.2	Xenobiotic metabolism	4.8
KRT7*	Keratin 7	12q13.13	Cytoskeletal filament	6.5
LMO3	LIM domain only 3	12p12.3	Unknown	4.2
ME1	Malic enzyme 1, NADP(+)-dependent, cytosolic	6q14.2	Fatty acid metabolism	4.6
PKD4	Pyruvate dehydrogenase kinase, isozyme 4	7q21.3	Sugar metabolism	3.8
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	10p15.1	Sugar metabolism	4.1
PTGS2	Prostaglandin-endoperoxide synthase 2	1q31.1	Prostaglandin synthesis	4.5
PYGL*	Phosphorylase, glycogen	14q22.1	Glycogen metabolism	4.0
SERPINA5	Serpin peptidase inhibitor, clade A, member 5	14q32.13	Coagulation	77.1
SLFN11	Schlafen family member 11	17q12	Unknown	3.3
SLPI*	Secretory leukocyte peptidase inhibitor	20q13.12 [#]	Immune response	13.5
SNCG	Synuclein, gamma	10q23.2	Neurofilament network	3.8
SPINT1*	Serine peptidase inhibitor, Kunitz type 1	15q15.1	Protease inhibitor	3.4
TGFB3	Transforming growth factor, beta receptor III	1p22.2-p22.1	TGFbeta signaling	4.1

* Genes upregulated in cultured prostate cancer cells following 5-aza-dC treatment.

[#] Cytobands coinciding with prostate cancer susceptibility loci.

≠ Average ratio of expression in seminal vesicle compared to normal prostate

Table 2

Candidate prostate cancer genes (increased in prostate vs. seminal vesicle)

Gene symbol	Gene name	Cytoband	Biologic process	PN/SV [≠]
CENPN	Centromere protein N	16q23.2	Cell division	5.5
ENTPD5	Ectonucleoside triphosphate diphosphohydrolase 5	14q24.3	Nucleotide catabolism	11.4
GOLM1	Golgi membrane protein 1	9q21.33	Golgi transport	8.3
GPR160	G protein-coupled receptor 160	3q26.2	Unknown	4.0
GUCY1A3	Guanylate cyclase 1, soluble, alpha 3	4q32.1	Signal transduction (nitric oxide)	4.5
KCNN2	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	5q22.3	Membrane conductance	4.9
LOC202451	Hypothetical protein LOC202451	6q23.3	Unknown	6.4
LRIG1	Leucine-rich repeats and immunoglobulin-like domains 1	3p14.1	Regulation of cell proliferation	5.2
SIM2	Single-minded homolog 2	21q.22.13	Transcriptional regulation	3.6
SPOCK1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	5q31.2	Unknown	4.4
TARP	TCR gamma alternate reading frame protein	7p14.1	Unknown	37.8

[≠] Average ratio of expression in normal prostate compared to seminal vesicle.