Differential gene expression in tumor adjacent histologically normal prostatic tissue indicates field cancerization

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Abstract. Field cancerization denotes the occurrence of aberrant cells in tumor adjacent histologically normal tissues (TAHN). To characterize field cancerization in prostate cancer, we used RNA from paired patient tumor and TAHN tissues excised at 1 cm from the tumor margin and subjected them to microarray expression analysis comparative to RNA from normal cancer-free prostatic tissues. Eleven novel transcripts were significantly up-regulated in TAHN tissues and also in tumors. Expression of early growth response protein 1, tristetraprolin, testican, and fatty acid synthase, mutually up-regulated at different levels in tumors and TAHN tissues was confirmed by quantitative reverse transcriptase PCR in the experimental and in an independent validation set. This study offers proof of expressional changes in field cancerized prostatic TAHN tissues at defined distances from tumor margins. Markers of field cancerized prostatic tissues could be early diagnostic indicators in biopsies after abnormal prostate-specific antigen and digital rectal examination and independent of cancerous histology and/or early targets for chemo-preventive intervention in pre-malignant disease.

Introduction

The terms 'field cancerization' or 'field effect' were first introduced in tumors of the head and neck to describe the occurrence of genetic alterations in histologically normal tissues adjacent to tumors (1-4). Such alterations outside the histologically visible tumor margins could result from pre-existing fields of genetically compromised cells in which the tumor develops. Alternatively, the tumor could influence the surrounding tissue, or it may reflect a combination of these two scenarios. While the underlying mechanisms of field cancerization remain unclear, its occurrence has been described in several epithelial cell derived tumors, including but not limited to lung, esophageal, colorectal, breast and skin cancers (1,4,5). In contrast, relatively little is known about field cancerization in prostate cancer, perhaps due to its previously reported multifocal nature (5,6). In addition, prostate cancer is often present in the setting of other benign prostatic conditions, most frequently benign prostatic hyperplasia (BPH), which could influence adjacent cells and thus affect the characterization of field cancerized tissue. Finally, due to the relatively small size of the human prostate, the entire organ may be affected, either genetically or biochemically, excluding the existence of matched, truly normal, i.e. entirely unaffected tissue from the same patient.

Field cancerization is of clinical importance (5). In prostate cancer, markers of field cancerization may be important for confirming or detecting disease in biopsies after abnormal prostate-specific antigen (PSA) and/or digital rectal examination (DRE), the current standard of care for detecting prostate cancer. PSA screening has led to earlier detection and an overall decrease in prostate cancer-specific mortality, emphasizing the importance of prostate biopsies (7,8). However, biopsy tissue represents a very small portion of the prostate and consists primarily of tumor adjacent histologically normal (TAHN) tissue. In spite of ultrasound guidance, it is easy to miss a small focal malignancy. The current accuracy of prostate cancer detection/confirmation by biopsy is ~25% with the rest representing false-negative diagnoses (9,10). In the presence of an abnormal PSA and/or DRE, this represents a dilemma for the patient and his physician. Therefore, biomarkers that are indicative of disease, yet independent of cancerous histology, i.e. present in field cancerized TAHN tissue, could greatly increase the accuracy of early prostate cancer detection in biopsies (5).

Our laboratory has previously investigated the nature of field cancerization in both prostate and breast cancers using markers of genomic instability, including telomere DNA content (TC), an established surrogate measure of telomere length and the extent of allelic imbalance (AI) (11,12). These studies have shown telomere alterations and the presence of AI in both tumor and TAHN tissues. In particular, alterations

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in TC seen in prostate tumors were frequently mimicked in the matched TAHN tissues, indicating prostatic field cancerization (11). Based on these observations, we hypothesized that the molecular changes would not be limited to genomic instability, but may include consistent alterations in gene expression. To test this hypothesis, we have conducted a study utilizing microarray expression analysis of cancerous and TAHN prostatic tissues isolated at 1 cm from the visible tumor margin. We report here the identification of consistently altered gene expression in TAHN tissues indicative of field cancerization in prostate cancer.

Materials and methods

Prostate sample collection, preparation and demographics. Twelve matched prostate tumors and TAHN tissues excised at 1 cm from the visible tumor margin (~150 mg each), resulting in a total of 24 samples, were obtained from the University of New Mexico Hospital Pathology Laboratory in agreement with all University, State and Federal laws. The median age of the cohort was 57 years with a range of 51-71 years; all samples had Gleason scores of 3+3 or 3+4 and a stage of T2 with the exception of two T3 cases; all samples were node-negative (Table I). Tissue samples were snap-frozen in liquid nitrogen immediately after collection and stored at -70˚C. A portion of the frozen tissues (~50 mg) was homogenized and RNA was isolated and resuspended in RNase-free water (Qiashredder and RNeasy kits Qiagen, Valencia, CA). In order to characterize the tissue portions used for RNA isolation by histopathology, directly adjacent tissue portions (~50 mg per sample) were formalin-fixed and paraffin-embedded for sectioning and hematoxylin and eosin (H&E) staining for independent pathological review (Fig. 1). Samples were randomized into 2 groups, the microarray set (MA set, Table I) and the validation set (VA set, Table I). Each group consisted of 6 patient matched tumor and TAHN samples; the MA samples were those designated 1-6, while the VA set were the samples designated 7-12 in Table I. For the six cases chosen for microarray analysis, a total of 1 μg of the isolated RNA was pooled to generate the MA set, while the remaining RNAs and RNAs from six additional cases (independent VA set) were stored separately.

Six prostate samples from cancer-free controls (unrelated death cases) were obtained from the National Cancer Institute Cooperative Human Tissue Network (CHTN; Nashville, TN), stored at -70˚C and subjected to RNA extraction and histological review. The latter confirmed these samples to be cancer-free and also free of BPH (Fig. 1). The median age of this set was 44.5 years, with a range of 26-79 years (Table I).

Microarray expression analysis. RNA integrity was analyzed using the Agilent Bioanalyzer 2100 (Agilent, Foster City, CA). RNAs from six matched tumor and TAHN tissues were selected to be prepared for microarray analysis based on RNA quality and quantity (the MA set). RNA from the selected samples was combined in equal parts to a total of 1 μg to generate the tumor and TAHN pools for the MA set. Control RNA for microarray analysis was obtained from Ambion (Austin, TX) as a customized service and consisted of RNA pooled from 9 deceased organ donors of Caucasian descent.

Table I. Description of prostate samples used in this study.[a]

Sample	Patient's age	Gleason score[b]	TNM stage[b]
Tumor/TAHN			
1	58	3+3	T3/III
2	57	3+3	T2/II
3	71	3+4	T3 N0/III
4	64	3+3	T2a N0/II
5	53	3+3	T2c N0/II
6	57	3+4	T2c N0/II
7	51	3+4	T2c/II
8	60	3+4	T2a N0/II
9	50	3+3	T2c N0/II
10	55	3+3	T2c/II
11	64	3+3	T2 N0/II
12	53	3+4	T2c/II
Normal			
13	46	na[c]	na
14	55	na	na
15	43	na	na
16	79	na	na
17	26	na	na
18	43	na	na

[a]The cohort consisted of i) 12 tumor and matched tumor adjacent histologically normal (TAHN) human tissues collected at the University of New Mexico Health Sciences Center and ii) 6 normal, cancer-free prostates obtained from the Cooperative Human Tissue Network. [b]Tumor Nodes Metastasis (TNM) stage was assigned using criteria published by the American Joint Committee on Cancer (http://www.cancerstaging.org/index.html). Gleason scores and stages were determined from the prostatectomy samples. [c]Not applicable.

Great care was taken in choosing cases with a similar age range (median age was 70 years, the range was 45-79 years), and cause of death unrelated to prostatic disease; in fact, the majority of cases were victims of myocardial infarction and cardiac arrest. RNA was reverse transcribed into complementary DNA (cDNA) using the Retroscript™ RT kit (Ambion), followed by labeling with either Cy3 (pooled control RNA) or Cy5 (either tumor or TAHN pool) fluorescent cyanine dyes. Labeling was achieved by synthesizing the cDNAs in the presence of amino allyl dUTP (Sigma-Aldrich, St. Louis, MO) followed by chemically coupling of either Cy3 or Cy5 monofunctional dye (Amersham-Pharmacia Biotech, Arlington Heights, IL) to the cDNA. This process avoids biased incorporation of the dyes during reverse transcription.

Glass-slide-spotted-expression microarrays of the Qiagen Human Genome Oligo Set Version 3.0 (Qiagen) were used for this investigation. The arrays contained 37,123 transcripts, including 24,650 known genes, the rest being expressed sequence tags (ESTs) and controls. The design of these arrays is based on the Ensembl Human 13.31 Database (http://www.ensembl.org/) and on the Human Genome Sequencing Project. Equal parts of Cy3 and Cy5 labeled cDNAs were then combined and competitively hybridized to the microarray slides using the GeneTAC Genomic Solutions

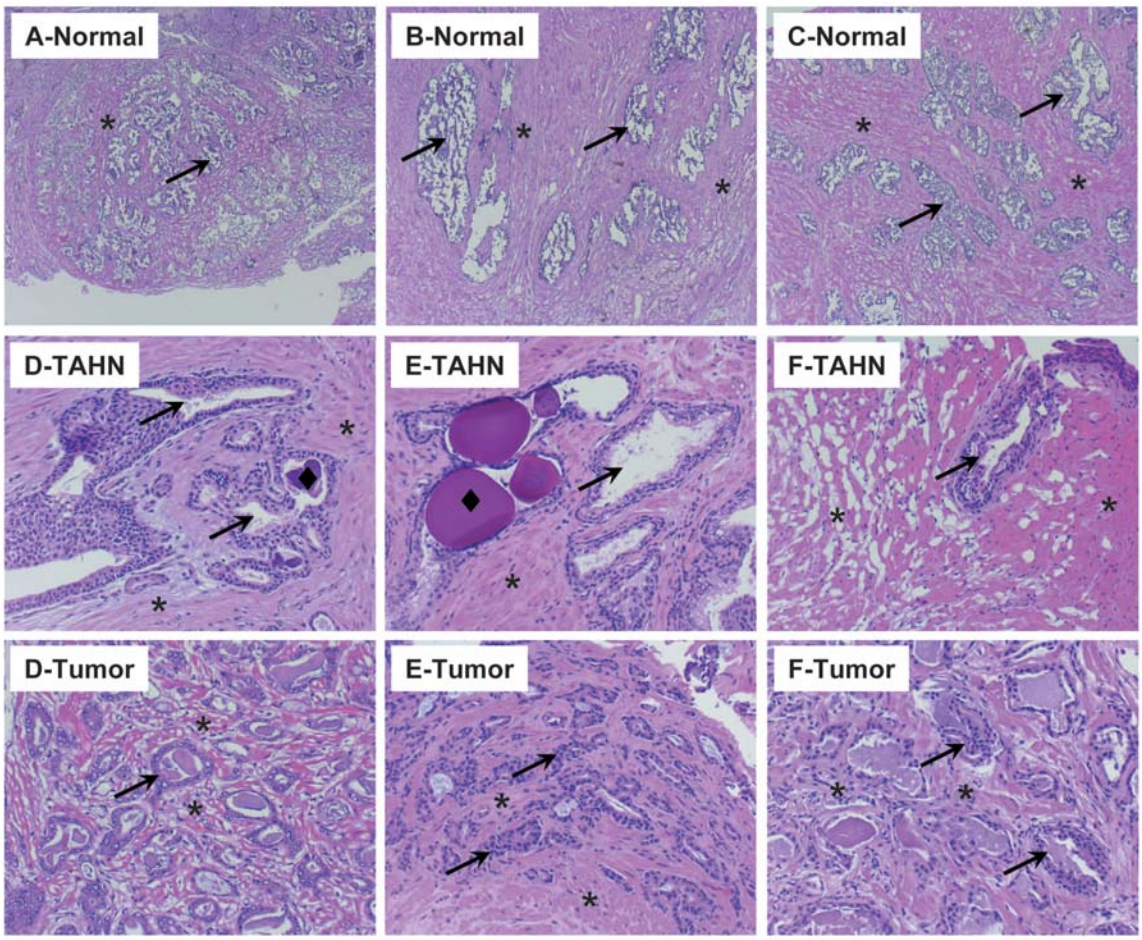

Figure 1. H&E staining of 3 cancer-free normal prostate tissues (A-C) and 3 representative cases of tumor and tumor adjacent histologically normal (TAHN) tissues (D-F, cases 5, 6, and 8 in Table I). (A-C) At x40 magnification; (D and E) at x200 magnification; arrows and asterisks denote glandular (ductal epithelial) and stromal areas, respectively; diamonds in D-TAHN and E-TAHN denote *corpora amylacea* (sedimented sulfated glycosaminoglycans) often seen in normal prostatic tissues (55).

machine and protocol (Genomic Solutions Inc, Ann Arbor, MI). Following hybridization and washing, the slides were scanned at 532 and 635 nm using the Axon 4000A scanner (Axon Instruments, Union City, CA) and the signal data were processed using Axon GenePix Pro 5 software (Axon Instruments). Fluorescence intensities of the Cy3 and Cy5 dyes were determined for each oligonucleotide spot, followed by visual inspection prior to importing into Acuity 3.0 (Molecular Devices, Sunnyvale, CA). This program was utilized to normalize the data and allow for comparison between the replicates using standard quality calls (background removal, linear regression ratio >0.6, signal to noise ratio >3.0). Only data passing these quality filters were utilized in the present analysis. Sample groups, i.e. tumor and TAHN pools, were run in triplicate hybridizations.

Quantitative (real-time) reverse transcriptase PCR. Quantitative real-time PCR (qRT-PCR) was used to verify the results of the microarray expression analyses. Samples from both the MA and the independent VA sets were individually analyzed in quadruplicate for each selected gene/primer set. Approximately 1 μg of RNA from the samples was converted to cDNA using the Retroscript™ RT kit (Ambion) according to the manufacturer's protocol using random decamers. The cDNAs were subsequently diluted 1:5 for use in the PCR reactions.

Genes included in mRNA expression evaluation included early growth response protein 1 (EGR-1), tristetraprolin (TTP), testican, fatty acid synthase (FAS), tissue inhibitor of metalloproteinase 2 (TIMP2) and superoxide dismutase 2 (SOD2). mRNA levels were quantitated using the SYBR-Green real-time PCR assay kit (Applied Biosystems, Foster City, CA) in a 25 μl reaction, using 0.5 μl of the diluted cDNA. Primers were used at a final concentration of 400 μM for both the forward or reverse in each reaction with the exception of EGR-1, for which the forward primer was used at a final concentration of 1 μmol, the reverse at a final concentration of 1.5 μmol in the PCR reaction. The primer sequences are listed in Table II. PCR reactions were carried out under the following cycling parameters: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min using the Gene Amp® 7000 Sequence Detection System (Applied Biosystems). Baseline fluorescence was determined during cycles 6-15.

The levels of EGR-1, TIMP2 and SOD2 were determined using the $\Delta\Delta$Ct method, where the threshold of detection of the genes of interest were compared to a house-keeping gene, either the TATA binding protein (TBP) (for EGR-1),

Table II. Primers used for qRT-PCR validation of microarray experiments.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product basepairs
Gene of interest			
EGR-1	GAGCAGCCCTACGAGCAC	AGCGGCCAGTATAGGTGATG	130
FAS	AGAACTTGCAGGAGTTCTGGGACA	TCCGAAGAAGGAGGCATCAAACCT	149
Testican	TGGAACCGCTTTCGAGACGATGAT	CACACACTTTGTGAGGGCTGCATT	124
TTP	GTTACACCATGGATCTGACTGCCA	AGTCCCTCCATGGTCGGATGG	86
TIMP2	TGCAATGCAGATGTAGTGATCAGGGC	GGGTTGCCATAAATGTCGTTTCCAG	80
SOD2	AGCATGTTGAGCCGGGCAGTGT	TGCTTCTGCCTGGAGCCCAGATAC	74
Loading control			
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC	112
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	70

Table III. Cy3/Cy5 fluorescent dye bias control microarray hybridization compared to experimental set using tumor and matched tumor adjacent histologically normal (TAHN) tissues.

	TAHN	Tumor	Cy3/Cy5 dye bias test
Mean ± SD[a]	1.58 ± 0.61	1.63 ± 0.75	1.27 ± 0.35
Median	1.49	1.51	1.22
Coefficient of variation (%)[b]	38.6[b]	46.1[b]	27.3

[a]Mean ± standard deviation (SD) for all transcripts detected. [b]Significant difference ($p<0.05$) from Cy3/Cy5 dye bias test.

or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (for TIMP2 and SOD2). This method was chosen because the amplification efficiencies of their primers were determined to be similar to the ones of the control transcripts. The remaining genes, i.e. FAS, TTP and testican, were evaluated using quantitation compared to serial dilutions of plasmids carrying cDNAs for these transcripts. Expression level calculations were controlled by the PCR efficiency corrected comparative quantitation method. Plasmids containing FAS, TTP, testican, and TBP PCR fragments were constructed using the pGem T-Easy vector (Promega Corporation, Madison, WI) and the PCR product incorporation was verified by sequencing. The data were reported as relative expression of genes of interest in tumor and TAHN RNA compared to expression levels in the pooled control prostate RNA.

Statistics. qRT-PCR results obtained from the microarray and validation sets were analyzed using JMP IN version 3.2.1 from Statistical Analysis Software (SAS; Cary, NC). Differences in the means between tumor or TAHN and cancer-free samples were analyzed using unpaired two sample t-test; differences between matched tumor and TAHN samples were analyzed using paired two sample t-test; differences with $p<0.05$ were considered statistically significant.

Results

Microarray expression analysis. We report RNA expression levels as ratios of Cy3/Cy5 signals for individual transcripts, where the Cy5 and Cy3 fluorescent cyanine dyes were used to label cDNA from experimental (tumor or TAHN) and pooled cancer-free control tissues, respectively. While a ratio of 1.0 would thus indicate no change in expression compared to cancer-free controls, there is the possibility of dye bias due to differential incorporation of Cy3 and Cy5 during cDNA synthesis, or due to differential hybridization of Cy3- and Cy5-labeled cDNAs to target probes. To estimate the extent of potential dye bias, we labeled paired aliquots of control cDNA from cancer-free prostatic tissues with Cy3 and Cy5, combined equal amounts of the preparations and hybridized them to a microarray set. Fluorescence analysis revealed a mean Cy3/Cy5 ratio of 1.27±0.35 standard deviation (SD), a median ratio of 1.22 and a coefficient of variation of 27.3% for all transcripts (Table III). In contrast, the mean ± SD and coefficients of variation determined for the TAHN and tumor experimental sets were 1.58±0.61 and 38.6% and 1.63±0.75 and 46.1%, respectively. Statistical analysis for the distribution of values for all detected transcripts revealed significant differences ($p<0.05$) for the tumor and TAHN microarray data from the Cy3/Cy5 dye bias test (Table III). While this result indicated a minimal dye bias for Cy3 fluorescent cyanine cDNA incorporation and/or target hybridization, we considered all transcripts in the experimental sets with an expression ratio of <1.27 as equally or underexpressed compared to normal cancer-free prostatic tissues in order to avoid false-positive assignment of up-regulated genes. Consideration of the Cy3/Cy5 dye bias is important because we focused our analyses of the microarray expression experiments on up-regulated transcripts, since overexpression of a protein marker

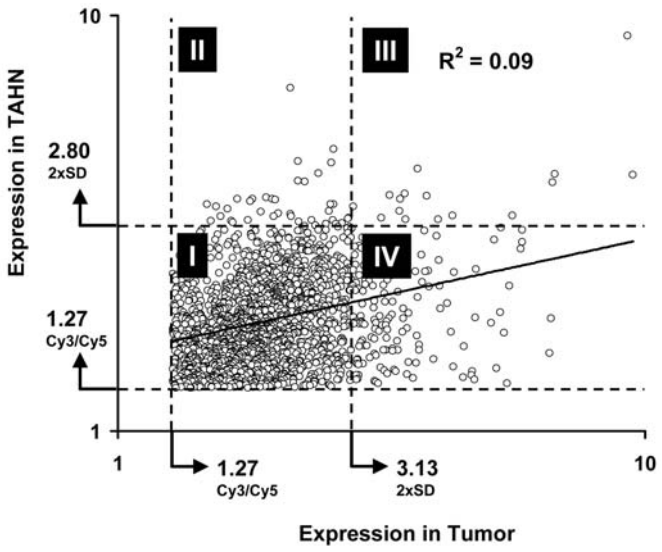

Figure 2. Analysis of microarray expression. Scatter plot of 1810 transcripts (open circles) mutually expressed at >1.27 compared to cancer-free prostatic samples in tumor and TAHN tissues as analyzed by microarray analysis (unknown transcripts included). Expression in tumor and TAHN tissues is shown on the log-scaled x-axis and y-axis, respectively. The Cy3/Cy5 dye bias and the 2xSD thresholds (as defined in Table III) are indicated by arrows and dotted lines. The solid line shows the best fit by logistic regression analysis accompanied by correlation coefficient R^2. Quadrant I, transcripts expressed at <2.0xSD of the mean expression (see Table III) of all transcripts expressed in tumor and TAHN tissues (i.e. <3.13 and <2.80, respectively); quadrant II, transcripts expressed at >2.0xSD of the mean expression in TAHN and at >1.27 in tumor tissues; quadrant III, transcripts expressed at >2.0xSD of the mean expression in both TAHN and tumor tissues; quadrant IV, transcripts expressed at >2.0xSD of the mean expression in tumor and at >1.27 in TAHN tissues.

in TAHN tissues would be amenable to positive identification and could thus be used in diagnostic tests.

In the microarrays, 3769 transcripts were mutually expressed in both tumor and TAHN tissues, 1810 of which were expressed above the Cy3/Cy5 dye bias of 1.27. We plotted the expression levels for these mutually expressed transcripts and analyzed their correlation between tumor and TAHN tissues (graphically shown Fig. 2). Logistic regression analysis indicated a correlation coefficient R^2 of only 0.09, indicating overall poor concordance of the expression levels between tumor and TAHN tissues. The majority of these transcripts, i.e. 94% were expressed at <2.0xSD of the mean expression (see Table III) of all transcripts expressed in tumor and TAHN tissues (i.e. <3.13 and <2.80, respectively), as shown in quadrant I of Fig. 2.

We used overexpression in the tumor tissues as a guide for the selection and further analysis of transcripts in the TAHN tissues. Accordingly, we identified the transcripts that were up-regulated in the tumor tissues at >2.0xSD of the mean, i.e. all transcripts with a ratio >3.13. Omitting expressed sequence tags (ESTs) and unknown open reading frames (ORFs), this identified 120 known transcripts up-regulated in tumor tissues. Of these, 97 transcripts were also expressed in the TAHN tissues, 70 of which were also expressed at >1.27, i.e. above the Cy3/Cy5 dye bias threshold (quadrants III + IV, Fig. 2). Eighty-three transcripts were up-regulated in the TAHN tissues at >2.0xSD of the mean, i.e. all transcripts with a ratio >2.80 (quadrants II + III, Fig. 2). We show the top 40 unique transcripts mutually up-regulated in tumor and TAHN tissues resulting from these analyses in Table IV. The number of mutually expressed and known transcripts at >2.0xSD for both tumor and TAHN tissues was 11 (quadrants III, Fig. 2).

qRT-PCR validation of microarrays. As shown in Fig. 2, microarray analysis indicated extensive heterogeneity of expression between tumor and TAHN tissues for the majority of transcripts. However, our microarray expression results represent mean values generated using pooled RNA populations. Therefore, it was important to estimate the extent of heterogeneity in individual samples. For this, we used qRT-PCR to test and validate the findings of the microarray expression analysis on selected transcripts in RNA samples of tumor and TAHN tissues compared to normal cancer-free prostate tissues. To better characterize the extent and heterogeneity of prostatic field cancerization in individual samples, we deliberately chose transcripts from above, below and at the 2.0xSD threshold of the mean in TAHN transcripts (i.e. ~2.8-fold up-regulated compared to cancer-free tissues, as defined in Table III). Early growth response protein 1 (EGR-1) represents the transcript most up-regulated (8.92-fold) in TAHN tissues and has been previously implicated in prostate tumorigenesis (13-20). Its expression in tumor tissue was 9.27-fold (Table IV). Testican, also known as SPOCK-1, was up-regulated at 4.29-fold and 1.73-fold in tumor and TAHN tissues, respectively. Testican has recently been shown to be expressed in prostatic tissues (21). Fatty acid synthase (FAS) represents an expected change in tumorigenesis of the prostate (22,23) and was up-regulated at 5.31-fold and 1.93-fold in tumor and TAHN tissues, respectively. In contrast, tristetraprolin (TTP) has not been previously reported to be associated with prostate tumorigenesis and may thus represent a novel finding. It was expressed at 5.81-fold and 2.75-fold in tumor and TAHN prostatic tissues, respectively (Table IV). For control purposes, we also included two transcripts that were equally or underexpressed in either tumor or TAHN tissues, i.e. tissue inhibitor of metalloproteinase 2 (TIMP2) and superoxide dismutase 2 (SOD2), expressed at 0.46-fold and 1.06-fold, and at 1.04-fold and 0.42-fold in tumor and TAHN tissues, respectively.

qRT-PCR validation was first performed on the six individual RNA samples pooled and used in the microarray expression analysis, the microarray (MA) set (Fig. 3). In this analysis, the expression levels were compared to 6 normal cancer-free prostate control samples. Although variation was observed, mean expression of FAS, TTP, EGR-1 and testican in TAHN tissues was significantly different from normal controls ($p<0.05$; p-range = 0.01-0.03). Similarly, mean expression for these transcripts in tumor tissues was significantly different from normal controls ($p<0.05$; p-range = <0.01-0.03). In contrast, and as expected, mean expression of the control transcripts TIMP2 and SOD2, which were equally or underexpressed in either tumor or TAHN tissues in the microarray experiments, was similar in TAHN and tumor tissues, as well as in normal controls ($p>0.05$; p-range = 0.27-0.70). Although not necessarily expected due to a higher degree of heterogeneity in cancerous tissues, expression of all of these transcripts was similar in TAHN

Table IV. Top 40 transcripts mutually up-regulated in tumor and corresponding matched tumor adjacent histologically normal (TAHN) tissues compared to normal cancer-free prostatic tissues.

Gene ID[a]	Gene description	TAHN[b]	Tumor[b]
H200019156	*Early growth response protein 1 (EGR-1)[c]*	***8.92[d]***	*9.27*
H200003548	Proto-oncogene protein c-Fos	**4.13[d]**	9.50
H200009720	Growth/differentiation factor 15 (GDF-15), macrophage inhibitory cytokine-1 (MIC1)	**3.96[d]**	6.68
H300013105	ETS-domain protein ELK-4	**3.70[d]**	3.28
H200005926	Metallothionein-1E (MT-1E)	**3.68[d]**	3.86
H300013389	Copine IV	**3.62[d]**	3.43
H300011237	Ergic-53-like protein precursor	**3.37[d]**	3.43
H300020290	Molecule possessing ankyrin repeats induced by lipopolysaccharide	**3.09[d]**	5.33
H300017466	Early response protein NAK1, TR3 orphan receptor	**2.94[d]**	4.03
H200000319	Aminopeptidase N	**2.85[d]**	5.85
H200019945	*Tristetraprolin (TTP)[c]*	***2.75[d]***	*5.81*
H300015296	Casein kinase I (CK1)	2.66	3.77
H200000676	Transcription factor Jun-D	2.64	3.45
H200006111	BTG2 protein	2.60	3.40
H200012441	Glandular kallikrein 1 precursor	2.58	4.06
H200020421	Paired immunoglobulin-like receptor ß	2.54	3.36
H300005679	Calreticulin precursor (CRP55), calregulin	2.45	4.79
H300014629	Tumor protein D52	2.43	3.88
H300022633	Similar to postmeiotic segregation increased 2-like 5	2.42	3.83
H300014182	Neprilysin	2.41	4.34
H300012307	Vascular endothelial growth factor A precursor (VEGF-A)	2.37	3.32
H300015765	Colorectal mutant cancer protein (MCC protein)	2.31	3.25
H300016106	Transcription factor EB	2.24	3.19
H300021922	Ubiquitin-protein ligase NEDD4-like	2.23	5.52
H300014306	HTPAP protein	2.23	4.08
H200014240	Poliovirus receptor related protein (CD112 antigen)	2.10	3.71
H300004950	Claudin-4	1.97	4.09
H300017343	*Fatty acid synthase (FAS)[c]*	*1.93*	*5.31*
H200017342	Prostein protein	1.92	3.56
H300005700	Keratin, cytokeratin 8 (CK 8)	1.90	3.31
H300012280	Prostate-specific antigen (PSA) precursor, kallikrein 3	1.89	3.15
H200003843	Diamine acetyltransferase	1.87	6.63
H300016780	Dolichyl-diphosphooligosaccharide-protein glycosyltransferas, 63 KD subunit	1.86	3.27
H200006197	NDRG1 protein	1.86	3.48
H300016292	Ubiquitin-conjugating enzyme E2-like	1.83	3.24
H200013682	X box binding protein-1 (XBP-1)	1.82	5.12
H300014868	KIAA0220-like protein (similar to nuclear pore complex interacting protein)	1.77	3.54
H300004833	*Testican (SPOCK-1)*	*1.73*	*4.29*
H200019551	Sialidase 1 precursor	1.72	5.40

[a]Gene identification number according to the Ensembl Human 13.31 Database (http://www.ensembl.org/). [b]Cy3/Cy5 ratios of tumor or TAHN (Cy5) compared to cancer-free normal (Cy3) tissues. [c]The 4 transcripts evaluated by qRT-PCR (Fig. 3) are in italics. [d]The bold represents transcripts above the 2xSD of the mean in TAHN tissues.

and tumor tissues ($p>0.05$; p-range = 0.07-0.59), with the exception of FAS ($p=0.02$). Thus, the results obtained with six individual RNA samples analyzed by qRT-PCR confirm the conclusions drawn from the analysis of pooled RNA by microarray expression analysis.

To corroborate these findings from the MA set, we also individually analyzed RNA from six independent tumors and patient matched TAHN tissues, the validation (VA) set. As in the MA set, mean expression of FAS, TTP, EGR-1 and testican in TAHN tissues was significantly different from normal controls ($p<0.05$; p-range = <0.01-0.03), demonstrating a consistent gene expression signature in TAHN tissues. In the VA set, mean expression of these transcripts in tumor tissues showed extensive variation when compared to normal controls, with EGR-1 and TTP showing significant and near significant differential expression ($p<0.01$ and $p=0.06$, respectively), and FAS and testican showing similar expression ($p=0.10$ and $p=0.27$, respectively). As expected, the control transcripts TIMP2 and SOD2 showed similar expression in TAHN and tumor tissues, and in normal controls ($p>0.05$; p-range = 0.28-1.00). Collectively, the qRT-PCR data (Fig. 3) was in excellent agreement with the data from the microarrays,

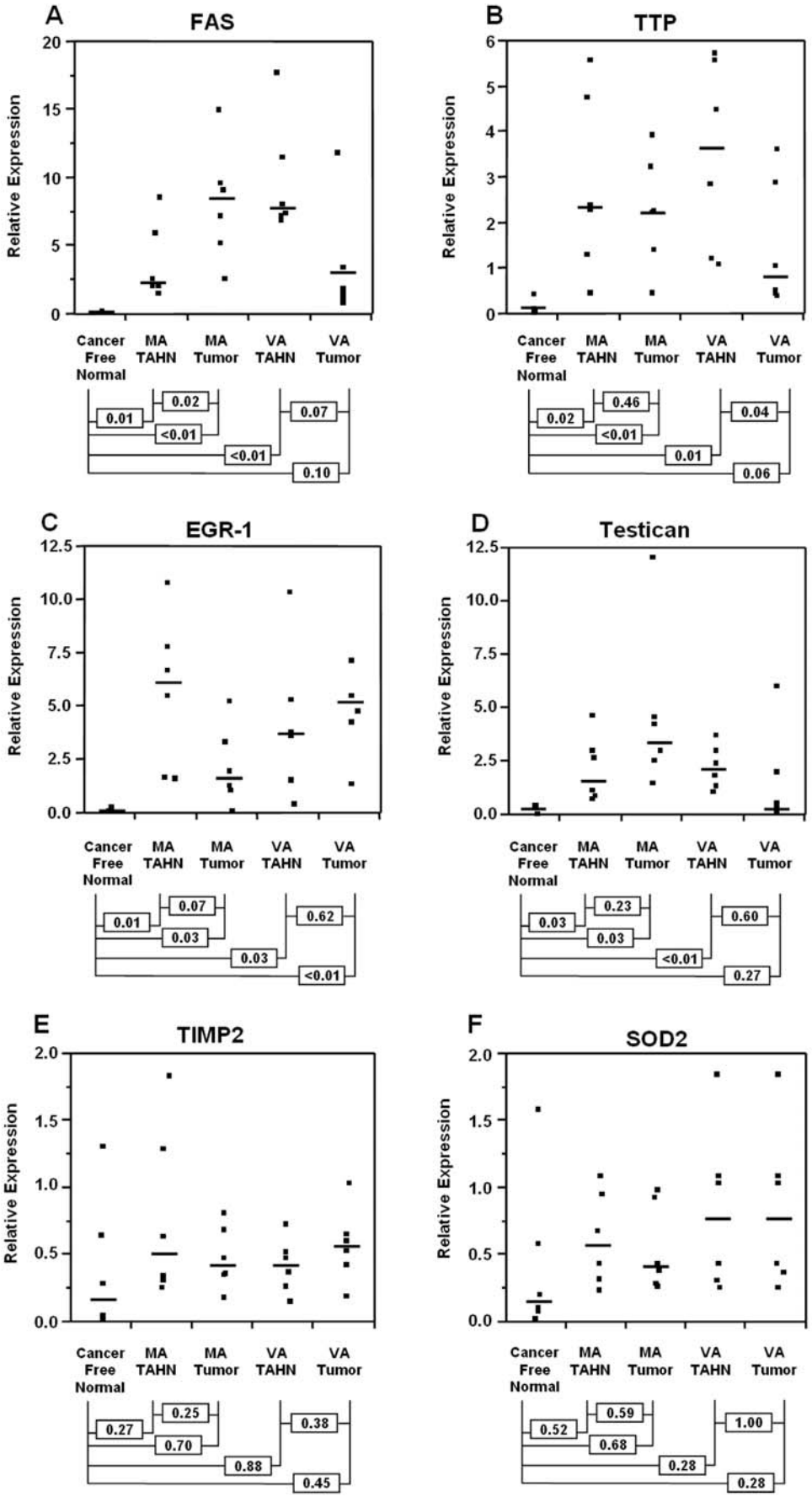

Figure 3. RNA expression levels by qRT-PCR of FAS (A), TTP (B), EGR-1 (C), testican (D), and the control transcripts TIMP2 (E), and SOD2 (F) normalized to either GAPDH or TBP. The tissue groups are indicated on the y-axis (MA, microarray set; VA, validation set; TAHN, tumor adjacent histologically normal). Expression is shown on the y-axis relative to cancer-free normal prostatic tissues, dots represent the distribution, and the horizontal line indicates the median. The numbers represent the p-values for differences between indicated groups as determined by the unpaired (compared to cancer-free tissues) and paired (compared to matched tissues) t-test.

thereby indicating the occurrence of field cancerization for the selected transcripts in TAHN when compared to tumor and cancer-free tissues.

Discussion

The major finding of this study is the occurrence of up-regulated transcripts in tumor adjacent histologically normal (TAHN) human prostatic tissues, as shown by microarray and qRT-PCR expression analysis of 12 mostly early stage (T2-T3) and low grade (Gleason sum 6-7) prostate tumors. While the study was not designed to provide a comprehensive signature of prostatic TAHN tissues, it does provide a strong indication of prostatic field cancerization and emphasizes its potential as a source of markers to be further verified in larger cohorts. We focused on the identification of transcripts that were up-regulated in both tumor and TAHN prostatic tissues, as such transcripts may have important clinical applications, especially for the alternative or adjunct diagnosis of prostatic malignancy after inconclusive or false-negative biopsy assessment. Along this line, Dhir and colleagues have reported on the identification of early prostate cancer antigen (EPCA), a biomarker that is expressed throughout the prostate of individuals with prostate cancer but not in those without the disease, also indicating field cancerization (24). The authors of that study showed that detection of EPCA resulted in minimal overlap between samples from patients with prostate carcinoma and controls and reported the identification of individuals with prostate cancer earlier than currently used diagnostics.

Several expression studies have reported unique molecular signatures for prostate cancer by comparing cancerous to histologically cancer-free adjacent tissues and attempting to link the gene profiles to clinicopathological patient information such as stage and Gleason sum scoring (25-32). However, the use of matched tissues as appropriate controls has been questioned due to field cancerized cells harboring genetic and biochemical alterations (33). This is supported by our prior (11,12) and present results. In contrast, few expression studies have reported molecular signatures and individual markers characteristic of prostatic TAHN tissues. Field cancerization is however evident at the genetic as well as the epigenetic level, as we have shown by altered telomeres in whole tissue TAHN extracts (11) and as shown by others by gene promoter methylation of APC, RARß2 and RASSF1A (34). Field cancerization in prostatic tissues is also evidenced by RNA/protein expression analysis (35,36). In a similar study, but without validation by qRT-PCR, Chandran and colleagues reported differentially expressed transcripts when comparing tumor associated matched tissues to cancer-free controls utilizing an Affymetrix platform (35). Of note, the authors claim that these transcripts would not be identified as differentially expressed when compared to tumors. Due to different platforms, patient populations, and sample preparations, it is difficult to compare findings between studies. For example, while the exact distance of TAHN tissue from the tumor is not known in most published studies, we have carefully chosen a defined distance of 1 cm. Despite differences, similarities reported by different groups corroborate the occurrence of field cancerization. Accordingly, Yu and colleagues previously showed not only expressional differences between tumor and matched tissues, but also between prostatic TAHN and tissues from cancer-free control donors (36). Of interest in their study, the transcription factor c-Fos was up-regulated 2.55-6.80 and 4.67-6.67 in TAHN and tumor tissues, respectively. This is similar to our own findings of 4.13-fold and 9.50-fold overexpression in TAHN and tumor tissues, respectively (Table IV). We also conducted a detailed review of the Gene Expression Omnibus (GEO) microarray data sets from the National Center for Biotechnology Information (NCBI) available at http://www.ncbi.nlm.nih.gov/geo/. We thereby focused on our top transcripts up-regulated in prostatic TAHN tissues, as well on the transcripts validated by qRT-PCR (Table IV). This review revealed one data set, GSE6919 that matched our criteria of comparing cancer-free normal, TAHN and tumor tissues (primary and metastatic) with similar tissue collection procedure and pathological assessment to exclude any obvious neoplastic alterations in TAHN and normal tissues. Despite similar heterogeneity, expression of EGR-1, c-Fos, FAS, GDF-15, and testican were up-regulated in tumors (2.5x, 5.9x, 2.3x, 1.2x, and 1.6x, respectively), and EGR-1, c-Fos, FAS, and testican were also up-regulated in TAHN tissues (2.3x, 6.7x, 1.3x, and 1.5x, respectively). Thus, for the most part, these comparisons validate our findings, and lead us to conclude that field cancerization is a rather strong and robust manifestation in prostatic tumors.

In the present study, we chose to use bulk tissue that was not microdissected in order to include both glandular (epithelial) as well as stromal (fibroblastic) compartments. While prostate adenocarcinoma is ultimately an epithelial disease, it is widely accepted that the stroma is involved in initiating, maintaining, and promoting a malignant phenotype through inter-cell signaling (37,38). These processes may also occur in TAHN tissues, as shown by Hanson and colleagues, who have reported promoter methylation for GSTP1, RARß2 and CD44 in stromal cells associated with tumors (39). Additionally, this approach also demonstrates that the identified gene expression changes could potentially be identified in biopsy samples. In the present study, we pooled samples for the microarray analysis in order to minimize effects of sample heterogeneity. The authenticity of our findings, however, was confirmed by qRT-PCR using RNA from individual samples. Although heterogeneity from patient to patient was observed, data validity was corroborated in an additional independent set of patient samples. The up-regulated transcripts observed in both tumor and TAHN tissues were identified in comparison to a mutually shared and appropriate background control, i.e. prostatic tissues from cancer-free deceased individuals with a similar age range.

Table IV indicates part of a signature that may be characteristic of prostatic field cancerized tissues. It is conceivable that some of the listed transcripts could have an important role in prostatic TAHN tissues, either a causative one as drivers of pre-malignancy or as a reaction to the presence of the tumor, or both. Among the 11 highest up-regulated transcripts in TAHN tissues were EGR-1, c-Fos, and the growth/differentiation factor 15 (GDF-15), also called macrophage inhibitory cytokine-1 (MIC1). EGR-1 has been strongly implicated in prostate cancer (13-20) and regulates

multiple target genes that in turn have a potential role in prostatic carcinogenesis and progression, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor (PDGF), and human telomerase reverse transcriptase (hTERT), thereby regulating a spectrum of cellular responses, including growth and growth arrest, survival and apoptosis, and differentiation and transformation (40,41). The involvement of c-Fos as part of the transcription factor activator protein 1 (AP-1) that is activated downstream of many growth factors is supported by a large body of literature on oncogenesis and metastasis (42,43). GDF-15 (MIC1) is a member of the transforming growth factor ß (TGFß) family and is known to be up-regulated in prostate cancer (44,45). In addition, increased levels of GDF-15 have also been correlated with metastasis and the development of sclerotic bone lesions, which are typical for prostate cancer (45). It has also been shown to contribute to chemotherapeutic drug resistance (46). Important to this study, GDF-15 expression was previously detected in benign tissue areas of prostate cancers affected with Gleason sum scores 5-8 disease (47). Similarly, elevated expression of metallothioneins (MT) was recently shown in tissues adjacent to head and neck cancers and breast adenocarcinomas, especially in the presence of affected lymph nodes (48), leading these authors to speculate that MT expression in normal tissues signals the presence of a tumor.

To further estimate the extent and heterogeneity of prostatic field cancerization, we included transcripts that were less up-regulated in TAHN tissues, such as TTP, FAS, and testican. TTP expression is not specific to prostatic tissues. However, it is a ubiquitously expressed AU-rich element (ARE) binding protein and a regulator of mRNA stability, including of pro-inflammatory proteins, such as tumor necrosis factor α (TNFα) (49), which plays an important role in prostate adenocarcinoma (50). It is possible that TNFα is produced by inflammatory cells in TAHN tissues in agreement with the prominent role of inflammation as proposed by De Marzo and colleagues (51). TNFα is a classical activator of the nuclear factor κ B (NFκB) pathway which is constitutively activated in prostate cancer with prominent downstream targets that support an activated cellular state, including EGR-1 (40,52). FAS has been termed a 'metabolic oncogene' and may reflect a prostate cell's energetic switch to a more anaerobic yet more reductive physiologic state, which is a hallmark of prostate cancer progression (22,23). In addition, FAS has been shown to positively affect NFκB nuclear translocation in cancer cells leading to an anti-apoptotic effect (53). Finally, testican (SPOCK-1) belongs to the fibulin protein family of extra-cellular matrix proteins which influence cell adhesion and migration, and have thus been associated with progression of several cancer types (54), including prostate cancer, in which it has recently been shown to be up-regulated (21).

Collectively, our data add to the rather scarce literature that supports field cancerization in prostatic tissues. Our findings warrant further investigations in larger cohorts of tissue samples collected at defined distances from tumor margins (1 cm in this study) and into its underlying mechanisms, as well as potential clinical use of representative transcripts towards an improved prostate cancer detection and patient outcome.

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