[CANCER RESEARCH 61, 5692–5696, August 1, 2001]

Advances in Brief

Expression Profiling Reveals Hepsin Overexpression in Prostate Cancer[1]

Jeffrey A. Magee, Toshiyuki Araki, Sushama Patil, Torsten Ehrig, Lawrence True, Peter A. Humphrey, William J. Catalona, Mark A. Watson, and Jeffrey Milbrandt[2]

Department of Pathology, Divisions of Laboratory Medicine [J. A. M., T. A., S. P., T. E., P. A. H., M. A. W., J. M.] and Department of Surgery, Division of Urology [W. J. C.], Washington University School of Medicine, St. Louis, Missouri 63110, and Department of Pathology, University of Washington Medical Center, Seattle, Washington 98195 [L. T.]

Abstract

Prostate cancer is the most commonly diagnosed noncutaneous cancer in men. Despite this fact, many of the genetic changes that coincide with prostate cancer progression remain enigmatic. We have addressed this problem by characterizing the expression profiles of several benign and malignant human prostate samples, and we have identified several genes that are differentially expressed between benign and malignant glands. One gene that was overexpressed encodes the serine protease hepsin. We used an independent sample set to confirm that hepsin is overexpressed in prostate tumors, and *in situ* hybridization demonstrates that hepsin is specifically overexpressed in the carcinoma cells themselves. These facts, together with the molecular properties of hepsin, make it an ideal target for prostate cancer therapy.

Introduction

Prostate cancer poses a significant clinical challenge both in terms of its prevalence and its complexity. In the year 2001, ~198,000 new cases of prostate cancer will be diagnosed in the United States, and ~32,000 American males will die of this disease (1). These patients will harbor heterogeneous tumors that dramatically vary in their rate of progression and their response to therapy. The scope of this problem has prompted intense scrutiny into the molecular genetics of prostate cancer. Recent studies have identified the PTEN, p27, and Nkx3.1 gene products as tumor suppressors in prostate epithelia (2, 3). Moreover, the well-characterized oncogenes c-*myc* and *bcl*-2 are commonly overexpressed in prostate tumors (2). Efforts to identify additional prostate cancer-related genes will be facilitated by microarray-based gene expression analyses used previously to analyze other tumor types. For example, Golub *et al.* (4) have used microarrays to discriminate between acute myeloid leukemias and acute lymphoblastic leukemias according to their expression profiles. More recently, microarray analysis has been used to characterize the molecular profiles of non-Hodgkin's lymphomas, breast cancer, colon cancer, and glioblastomas (5–8). Here we present the expression profiles of prostate tumor specimens of varying histological grades and clinical stages. By comparing the profiles of benign and malignant prostate specimens, we noted considerable heterogeneity between tumors but also identified several genes that are over- or underexpressed in a majority of tumors relative to normal prostate tissues. Among these tumor-associated genes was the gene *hepsin*. *Hepsin* encodes a *trans*-membrane serine protease that is also overexpressed in ovarian carcinomas and renal cell carcinomas (9, 10). Its overexpression in prostate cancer was confirmed molecularly on an independent panel of prostate specimens and by *in situ* hybridization. Thus, hepsin offers an enticing drug target for prostate cancer therapy.

Materials and Methods

Tissue Preparation and Probe Synthesis. Frozen tissue specimens were obtained from the Siteman Cancer Center Tissue Procurement Core at Washington University School of Medicine and from the University of Washington Medical Center under an Institutional Review Board-approved protocol. The specimens were blocked, and frozen sections were cut and analyzed by a single pathologist (P. A. H.) for Gleason grade and percentage of tumor. The tissues were then sectioned into Trizol (Life Technologies, Inc.) for RNA preparation. Antisense biotinylated riboprobes were synthesized from benign and tumor RNA samples as per the Affymetrix GeneChip protocol. Probes were then hybridized to Human 6800 Affymetrix GeneChips representing 7068 full-length human transcripts.

GeneChip Data Analysis. Average difference values from each individual chip were scaled such that the average intensity of any given chip was 1500. We discarded genes that were scored "absent" in every sample according to the Affymetrix Array Suite software package, leaving a filtered set of 4712 genes that were scored "present" on at least one chip. We established a lower boundary of 70.8 for the scaled average difference values of the remaining genes. This baseline represents the scaled noise of the "noisiest" chip included in the analysis.

To identify specific genes that were misexpressed in tumors relative to normal samples, we generated a computer algorithm allowing us to select genes exhibiting a ≥3-fold expression change in all of the 11 tumors relative to all of the four normal glands. We applied this algorithm to the scaled average difference values of the 4712-gene filtered data set. *P*s were calculated by a two-tailed *t* test for independent data sets of unequal size and variance.

Quantitative RT-PCR[3] Analysis. RNA was isolated from tissue samples as described above. Total RNA was reverse transcribed, and quantitative PCR was performed using the ABI sequence detection system 7700 machine. Amplicons were detected by SYBR green I fluorescence as described elsewhere (11). Data were analyzed with standard curves to determine relative gene expression levels.

***In Situ* Hybridization.** Sense and antisense digoxygenin-labeled RNA probes for *in situ* hybridization were transcribed from a fragment of the hepsin cDNA corresponding to nucleotides 1430–1730. The probe was hybridized to fresh frozen tissue samples as described previously (12). Briefly, frozen sections were fixed with 4% paraformaldehyde in 1 × PBS and treated with active 0.1% diethyl pyrocarbonate for 2 × 15 min. Probes were hybridized at 58°C for 40 h in 50% formamide/5 × SSC (pH 7.0). After washing, signal was detected by alkaline phosphatase-conjugated antidigoxigenin antibodies (Roche) followed by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results

Expression Profiling of Benign and Malignant Prostate Specimens. We analyzed the gene expression profiles of 11 malignant and four nonmalignant prostate samples. Tumor specimens included five low-grade primary tumors (Gleason grade 6), three high-grade samples (Gleason grade 7–9), one sample with local extraprostatic inva-

Received 5/1/01; accepted 6/15/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grant 5 P01 CA49712-08 and by grants from The Association for the Cure of Cancer of the Prostate (CaP CURE) and The Urological Research Foundation.

[2] To whom requests for reprints should be addressed, at Division of Laboratory Medicine, Department of Pathology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8118, St. Louis, MO 63110-1093. Phone: (314) 362-4650; Fax: (314) 362-8756; E-mail: jeff@pathbox.wustl.edu.

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; cdk, cyclin-dependent kinase; PIN, prostatic intraepithelial neoplasia.

sion into the seminal vesicle, and two lymph node metastases. To minimize the contributions of benign glands to the tumor profiles, we dissected the tumor samples to achieve >50% tumor within the sample for the primary tumors and 90–100% tumor for the metastases. We synthesized probes for the Affymetrix Hu6800 GeneChips and analyzed the expression data as described in the "Materials and Methods."

We generated a list of 4712 probe sets that were scored present on at least one chip by the Affymetrix Gene Expression Suite software. From this list, we sought to identify genes that are misregulated in tumors relative to benign specimens or misregulated in metastases relative to primary tumors. In comparing tumors with benign samples, we identified genes of which the scaled average difference values varied by ≥3-fold in all 11 tumor samples relative to all four of the benign samples (Table 1). In comparing metastases to primary tumors, we identified genes of which the scaled average difference values varied by ≥3-fold in all three of the metastases relative to all eight of the primary tumors (Table 1).

The initial comparison of tumors to benign glands revealed only a single up-regulated gene, *hepsin*, and no down-regulated genes (Fig. 1A). Therefore, we relaxed the stringency of our selection criteria to identify genes that exhibited a 3-fold expression difference in 9 of 11 tumors relative to all of the four benign samples. These criteria identified three additional misregulated genes (Fig. 1*B*). The serotonin receptor 2B and a cdk-related gene, *cdk10/PISSLRE*, were both up-regulated in tumors relative to benign glands, whereas the single down-regulated gene encodes a catalytically inactive phosphoglucomutase-related protein, Pgm-5. Statistically, the scaled average difference values of all four of the genes significantly varied between benign and tumor sample sets ($P < 0.05$). However, comparing expression levels in low-grade tumors to high-grade tumors to metastases did not reveal any correlations between pathological grade and gene expression (data not shown).

The comparison of metastases to primary tumors revealed three genes that were down-regulated in all three of the metastases relative to all eight of the primary tumors: *G protein α 16*, *pyridoxal kinase*, and *diacylglycerol kinase* (Table 1). We relaxed the selection criteria to include genes exhibiting a 3-fold difference among all three of the metastases and seven of eight tumor samples. These criteria identified

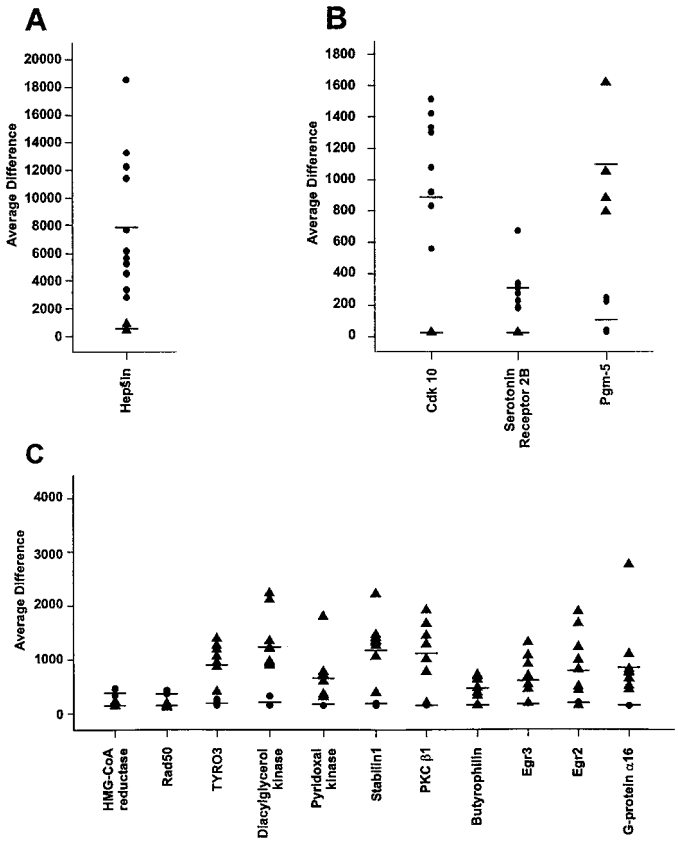

Fig. 1. Vertical scatter plots demonstrating absolute gene expression levels in individual tumors and benign glands from the initial microarray profiles. In all cases, the lower expression threshold is 70.8, representing the scaled noise of the noisiest GeneChip in the experiment. Scaled average difference values less than the threshold were adjusted to 70.8 and, therefore, overlap. *A*, genes that vary in tumors relative to normal glands. *B*, expression levels of Cdk10, serotonin receptor 2B, and Pgm-5 plotted with a reduced *Y* axis to better demonstrate their ranges of expression. *C*, genes that vary in metastases relative to primary tumors. Symbols correspond to specific pathological descriptions: *A and B*, ●, tumor; ▲, benign. *C*, ●, metastasis; ▲, primary tumor. *Horizontal bars* average gene expression in each respective sample set.

Table 1 *Misregulated genes in benign and malignant prostate samples*

	Accession	Mean avg difference	Mean avg difference	Mean fold difference	*P*
Gene[a]					
cdk 10	L33264	71 ± 0[b]	950 ± 519[c]	13.4	<0.001
Hepsin	X07732	186 ± 230[b]	7915 ± 4939[c]	42.5	<0.001
Serotonin receptor 2B	X77307	71 ± 0[b]	322 ± 161[c]	4.5	<0.001
Gene[d]					
Phosphoglucomutase 5	L40933	1136 ± 373[b]	109 ± 83[c]	10.4	0.011
Description[e]					
HMG-CoA reductase	M11058	80 ± 27[f]	335 ± 73[g]	4.2	0.021
Rad50	U63139	82 ± 24[f]	333 ± 36[g]	4.1	0.003
Gene[h]					
TYRO3 protein tyrosine kinase	U18934	952 ± 301[f]	134 ± 57[g]	7.1	<0.001
Diacylglycerol kinase	X62535	1294 ± 527[f]	127 ± 97[g]	10.2	<0.001
Pyridoxal kinase	U89606	628 ± 487[f]	71 ± 0[g]	8.8	0.014
Stabilin 1/KIAA0246	D87433	1240 ± 506[f]	98 ± 24[g]	12.6	<0.001
Protein kinase C, β 1	X07109	1125 ± 541[f]	80 ± 16[g]	14.1	<0.001
Butyrophilin	U90552	390 ± 196[f]	71 ± 0[g]	5.5	0.002
Early growth response 3	X63741	596 ± 374[f]	71 ± 0[g]	8.0	0.005
Early growth response 2	J04076	881 ± 615[f]	101 ± 12[g]	8.7	0.009
G protein α 16	M63904	913 ± 752[f]	71 ± 0[g]	12.9	0.016

[a] Up-regulated in tumors relative to normal.
[b] Normal.
[c] Tumor.
[d] Down-regulated in tumors relative to normal.
[e] Up-regulated in metastases relative to primaries.
[f] Primaries.
[g] Mets.
[h] Down-regulated in metastases relative to primaries.

an additional six down-regulated genes and two up-regulated genes in the metastatic tumor samples (Table 1 and Fig. 1*C*). The list of up-regulated genes includes the cholesterol biosynthesis enzyme HMG-CoA reductase and the DNA repair enzyme Rad50. Among the nine down-regulated genes, TYRO3, Protein kinase C, β1, diacylglycerol kinase, and G protein α 16 have been implicated previously as regulators of intracellular signal transduction (13–16). The list of down-regulated genes also includes the immediate early genes *Egr-2* and *Egr-3*.

We next sought to confirm the observed gene expression differences between malignant and nonmalignant prostate samples on an independent sample set. We isolated total RNA from 13 independent primary tumors and 10 nonmalignant samples for validation of the observed hepsin, serotonin receptor 2B, cdk10/PISSLRE, and Pgm-5 profiles. We used quantitative RT-PCR to measure gene expression in the independent data set. As was seen in the initial set of samples, *hepsin* and *Pgm-5* were significantly misexpressed in tumor-containing samples relative to nonmalignant tissues (Fig. 2). From these profiles, we conclude that *hepsin* is indeed up-regulated in a majority of prostate tumors and that *Pgm-5* is down-regulated in a majority of prostate tumors.

Characterization of Hepsin Expression by *in Situ* Hybridization. We have demonstrated in independent sample sets, with two different methods of analyzing gene expression, that *hepsin* is overexpressed in prostate tumors. However, neither expression arrays nor quantitative RT-PCR can distinguish between stromal and epithelial gene expression. Therefore, we used *in situ* hybridization to characterize *hepsin* expression in benign glands, in primary tumors, and in PIN. Benign glands express *hepsin* at very low levels in the luminal epithelia (Fig. 3). As expected, tumors express *hepsin* at dramatically higher levels than benign glands, and this expression is localized to the carcinoma cells rather than the adjacent stroma. Interestingly, the PIN lesions examined in this study also expressed higher levels of *hepsin* than benign glands (Fig. 3, *C* and *D*). These results demonstrate that *hepsin* overexpression correlates with neoplastic transformation in the prostate and that it is expressed specifically in the transformed epithelial cells.

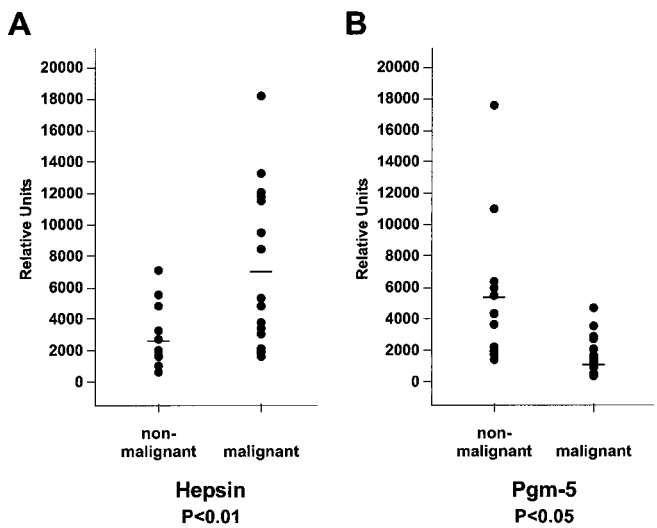

Fig. 2. Vertical scatter plots demonstrating relative expression levels for hepsin and Pgm-5 in an independent set of malignant and nonmalignant samples. By quantitative RT-PCR, these two genes both exhibited significant ($P < 0.01$ and $P < 0.05$, respectively) expression differences between malignant and nonmalignant samples in the validation sample set. *Horizontal bars*, average expression of hepsin and Pgm-5 in malignant and nonmalignant sample sets.

Discussion

The advent of microarray technology has prompted significant change in cancer research. By analyzing the expression of thousands of genes concurrently, the genetic changes that accompany tumorigenesis can be efficiently identified. We have used microarray-based expression profiling, quantitative RT-PCR, and *in situ* hybridization to identify and validate prostate cancer-associated genetic changes. Whereas these analyses yielded only two consistent, cancer-associated gene expression changes, we believe this reflects the histological and biological heterogeneity of the prostate cancer specimens. To minimize the occurrence of false positive genes, we selected genes that were misexpressed in >80% of tumors relative to all of the benign samples. However, given the fact that prostate cancer exhibits dramatic clinical variability and complex intratumoral cellular heterogeneity, we expect these samples to exhibit diverse molecular profiles. Indeed, hierarchical cluster analysis failed to segregate the prostate samples into meaningful clinical or pathological subgroups (data not shown). Furthermore, several prostate cancer-associated genes, including c-*myc*, *insulin-like growth factor-I*, *p27*, and *hepatocyte growth factor*, all displayed variable expression across all of the tumor samples.

One gene that is consistently down-regulated in tumor samples, *Pgm-5*, shares close homology with members of the phosphoglucomutase gene family. These genes encode proteins that convert glucose-1-phosphate to glucose-6-phosphate. However, Pgm-5 lacks a functional catalytic domain (17). Rather, Pgm-5 associates with the structural proteins dystrophin and utrophin, predominantly in smooth muscle cells (17). Whereas the observed difference between benign and tumor *Pgm-5* expression may simply reflect the altered cellular compositions of tumors, other smooth muscle markers such as smooth muscle myosin and dystrophin did not differ as dramatically or consistently. We suggest that the *Pgm-5* expression changes may reflect a *bona fide* smooth muscle response to tumorigenesis.

The most notable misregulated gene, *hepsin*, belongs to a family of trypsin-like transmembrane serine proteases that also includes TMPRSS2, corin, and enterokinase (14). Characteristic features of these type II integral membrane proteins include an extracellular serine protease domain, a scavenger receptor cysteine-rich domain, and an intracellular domain (14). Previous studies have reported *hepsin* expression predominantly in the liver, though low levels are also observed in the prostate gland (18). Interestingly, pathological overexpression of *hepsin* has also been reported in renal cell carcinoma and in most ovarian carcinomas (9, 10). These observations, together with our results, strongly imply a role for hepsin in the progression of several epithelial cancers.

Whereas the role of hepsin in prostate cancer progression awaits additional investigation, its homology to other serine proteases does offer tantalizing hypotheses. In general, extracellular proteases serve crucial functions in tumor progression, because they regulate growth factor availability and trigger the angiogenic switch. Furthermore, a related transmembrane serine protease, corin, cleaves and activates proatrial natruretic peptide (19). Thus, hepsin may cleave a currently unknown substrate, such as a growth factor propeptide, to promote tumor growth.

Several properties of hepsin make it an ideal target for prostate cancer therapy. The trypsin-like catalytic domain of hepsin is extracellular, thus facilitating drug delivery. Furthermore, the dramatic difference between hepsin expression in benign glands and malignant tissues suggests that prostate carcinomas would be acutely sensitive to hepsin inhibitors. Finally, hepsin-specific inhibitors should exhibit minimal toxicities, because hepsin-deficient mice lack an overt phenotype (20). Genetic studies with

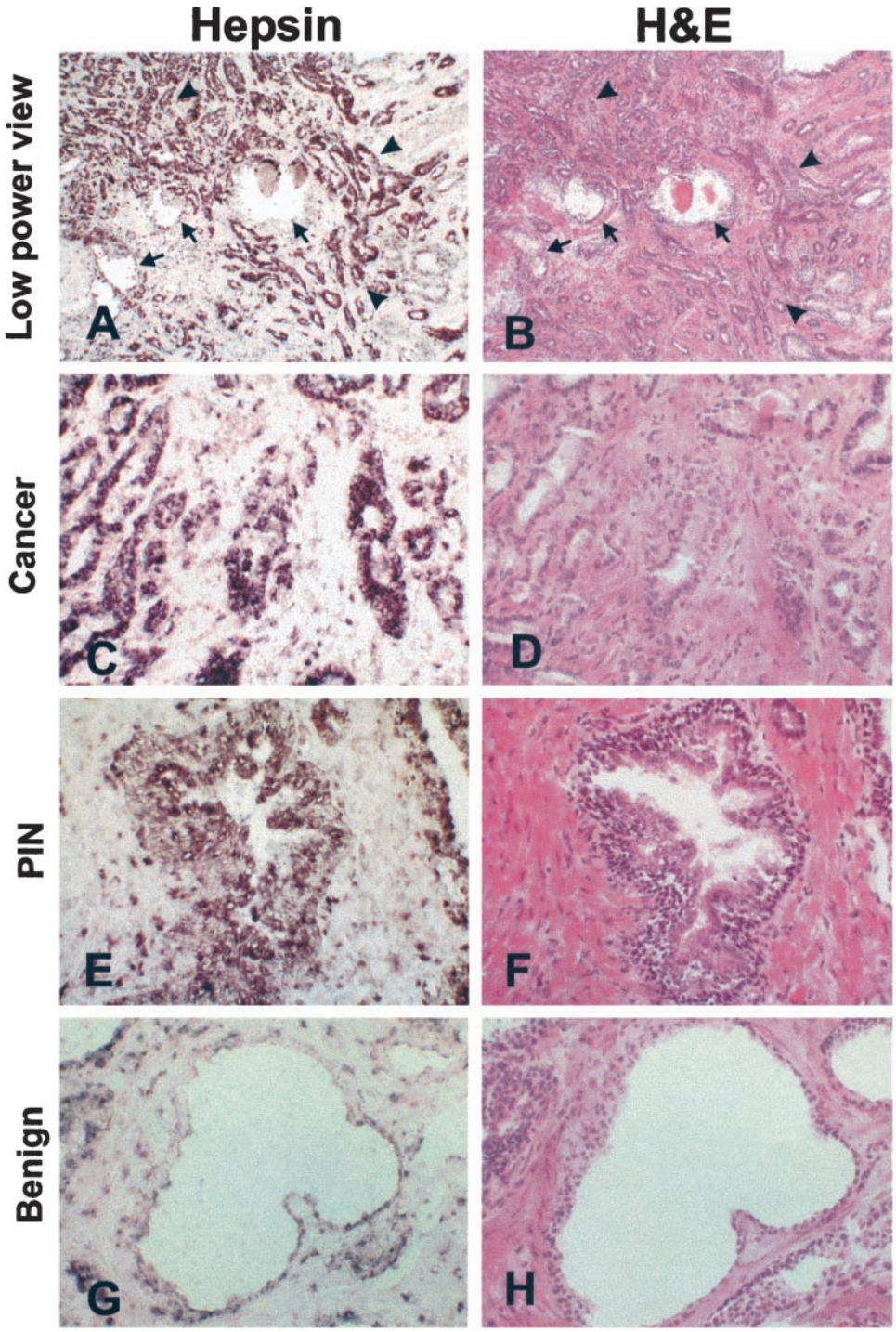

Fig. 3. *In situ* hybridization for hepsin expression in the prostate. H&E stains of an adjacent frozen section are shown at *right*. *A* and *B*, a low-power view of a prostate specimen with both benign (*arrows*) and malignant (*arrowheads*) epithelia. Hepsin is specifically overexpressed in malignant glands. *C* and *D*, a high-power view of malignant prostate epithelia overexpressing hepsin. *E* and *F*, a high-power view of PIN lesions that also overexpress hepsin. *G* and *H*, a high-power view of benign epithelia within the cancer specimen. Note that the benign glands to not express hepsin at appreciable levels.

mouse tumor models should help define the requirement for hepsin in tumor progression, and the genetic changes responsible for tumor-specific hepsin overexpression also warrant additional exploration. Given the known biological properties of hepsin and the several outstanding questions regarding its function in prostate cancer, the results presented here bring to light an exciting new avenue for the study and treatment of prostate cancer.

References

1. Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. Cancer statistics, 2001. CA - Cancer J. Clin., *51:* 15–36, 2001.
2. Abate-Shen, C., and Shen, M. M. Molecular genetics of prostate cancer. Genes Dev., *14:* 2410–2434, 2000.
3. Di Cristofano, A., De Acetis, M., Koff, A., Cordon-Cardo, C., and Pandolfi, P. P. Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. Nat. Genet., *27:* 222–224, 2001.
4. Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., Bloomfield, C. D., and Lander, E. S. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science (Wash. DC), *286:* 531–537, 1999.
5. Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, T., Yu, X., Powell, J. I., Yang, L., Marti, G. E., Moore, T., Hudson, J., Lu, L., Lewis, D. B., Tibshirani, R., Sherlock, G., Chan, W. C., Greiner, T. C., Weisenburger, D. D., Armitage, J. O., Warnke, R., Staudt, L. M., *et al*. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature (Lond.), *403:* 503–511, 2000.
6. Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lonning, P. E., Borresen-Dale, A. L., Brown, P. O., and

Botstein, D. Molecular portraits of human breast tumours. Nature (Lond.), *406:* 747–752, 2000.
7. Notterman, D. A., Alon, U., Sierk, A. J., and Levine, A. J. Transcriptional gene expression profiles of colorectal adenoma, adenocarcinoma, and normal tissue examined by oligonucleotide arrays. Cancer Res., *61:* 3124–3130, 2001.
8. Watson, M. A., Perry, A., Budhjara, V., Hicks, C., Shannon, W. D., and Rich, K. M. Gene expression profiling with oligonucleotide microarrays distinguishes World Health Organization grade of oligodendrogliomas. Cancer Res., *61:* 1825–1829, 2001.
9. Tanimoto, H., Yan, Y., Clarke, J., Korourian, S., Shigemasa, K., Parmley, T. H., Parham, G. P., and O'Brien, T. J. Hepsin, a cell surface serine protease identified in hepatoma cells, is overexpressed in ovarian cancer. Cancer Res., *57:* 2884–2887, 1997.
10. Zacharski, L. R., Ornstein, D. L., Memoli, V. A., Rousseau, S. M., and Kisiel, W. Expression of the factor VII activating protease, hepsin, *in situ* in renal cell carcinoma. Thromb. Haemostasis, *79:* 876–877, 1998.
11. Svaren, J., Ehrig, T., Abdulkadir, S. A., Ehrengruber, M. U., Watson, M. A., and Milbrandt, J. *EGR1* target genes in prostate carcinoma cells identified by microarray analysis. J. Biol. Chem., *275:* 38524–38531, 2000.
12. Braissant, O., and Wahli, W. A simplified *in situ* hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica, *1:* 10–16, 1998.
13. Lan, Z., Wu, H., Li, W., Wu, S., Lu, L., Xu, M., and Dai, W. Transforming activity of receptor tyrosine kinase tyro3 is mediated, at least in part, by the PI3 kinase-signaling pathway. Blood, *95:* 633–638, 2000.
14. Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., and Ullrich, A. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science (Wash. DC), *233:* 859–866, 1986.
15. Offermanns, S., and Simon, M. I. G α 15 and G α 16 couple a wide variety of receptors to phospholipase C. J. Biol. Chem., *270:* 15175–15180, 1995.
16. Schaap, D., de Widt, J., van der Wal, J., Vandekerckhove, J., van Damme, J., Gussow, D., Ploegh, H. L., van Blitterswijk, W. J., and van der Bend, R. L. Purification, cDNA-cloning and expression of human diacylglycerol kinase. FEBS Lett., *275:* 151–158, 1990.
17. Moiseeva, E. P., Belkin, A. M., Spurr, N. K., Koteliansky, V. E., and Critchley, D. R. A novel dystrophin/utrophin-associated protein is an enzymatically inactive member of the phosphoglucomutase superfamily. Eur. J. Biochem., *235:* 103–113, 1996.
18. Tsuji, A., Torres-Rosado, A., Arai, T., Le Beau, M. M., Lemons, R. S., Chou, S. H., and Kurachi, K. Hepsin, a cell membrane-associated protease. Characterization, tissue distribution, and gene localization. J. Biol. Chem., *266:* 16948–16953, 1991.
19. Yan, W., Wu, F., Morser, J., and Wu, Q. Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. Proc. Natl. Acad. Sci. USA, *97:* 8525–8529, 2000.
20. Wu, Q., Yu, D., Post, J., Halks-Miller, M., Sadler, J. E., and Morser, J. Generation and characterization of mice deficient in hepsin, a hepatic transmembrane serine protease. J. Clin. Investig., *101:* 321–326, 1998.

Expression Profiling Reveals Hepsin Overexpression in Prostate Cancer

Jeffrey A. Magee, Toshiyuki Araki, Sushama Patil, et al.

Cancer Res 2001;61:5692-5696.