

PSGR, a Novel Prostate-specific Gene with Homology to a G Protein-coupled Receptor, Is Overexpressed in Prostate Cancer¹

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Abstract

PSGR, a new prostate tissue-specific gene with homology to the G protein-coupled odorant receptor gene family, has been identified. Here we report the characteristics of the predicted protein sequence of *PSGR* and its prostate tissue specificity and expression profile in human prostate cancer and matched normal tissues. Using multiple tissue Northern blots from over 50 different tissues, *PSGR* expression was restricted to human prostate tissues. Paired normal and tumor specimens from 52 primary prostate cancers, obtained by laser capture microdissection or manual microdissection, were analyzed for *PSGR* expression by semiquantitative and real-time PCR assays. The differential expression of *PSGR* between normal and tumor tissues was highly significant ($P < 0.001$), and 32 of 52 (62%) matched prostate specimens exhibited tumor-associated overexpression of *PSGR*. Of note, there was very little or no expression of *PSGR* in many normal specimens in comparison with the generally high expression of *PSGR* seen in matched tumor specimens. *In situ* hybridization assays showed restricted *PSGR* expression in the epithelial cells of the normal and tumor tissue sections. Restricted expression of *PSGR* in prostatic epithelial cells, overexpression of the *PSGR* in a significant percentage of prostate cancers, and the predicted protein sequence of *PSGR* with seven transmembrane domains provide a foundation for future studies evaluating the potential of *PSGR* as a prostate cancer gene expression marker and the utility of *PSGR* protein as a novel target for developing immunotherapeutic strategies for prostate cancer.

Introduction

CaP⁴ is the most common malignancy in men in the United States and the second leading cause of cancer mortality (1). The serum PSA or HK3 test has revolutionized the early detection of CaP (2). Due to the prostatic epithelial cell-specific expression of PSA, it is also of value as a biomarker for disease remission/progression after treatment (2). Although PSA is effective at identifying men who may have prostate cancer, it is often elevated in men with benign prostatic

hyperplasia, prostatitis, and other nonmalignant disorders (2). Therefore, identification of additional prostate cancer-specific molecular markers, especially those exhibiting tumor-associated overexpression, is needed to refine the diagnosis as well as prognosis for CaP. It is also recognized that early detection of CaP presents new challenges with respect to predicting the clinical course of the disease for the individual patient (2). The wide spectrum of biological behavior exhibited by prostatic neoplasms calls for the identification of novel biomarkers that may be able to distinguish a slow-growing cancer from a more aggressive cancer with a potential to metastasize (2). Prostate cancer-associated molecular genetic alterations are being unraveled by using various strategies including: (a) analyses of genes commonly involved in human cancer; (b) positional cloning of putative genes on frequently affected chromosome loci in CaP; and (c) gene expression profiling of normal and tumor tissues from individuals with prostate cancer (3–5).

The discovery of additional prostate-specific genes has also resulted in enthusiasm for evaluating their potential utility in the diagnosis and prediction of disease progression of CaP. HK2, another member of the kallikrein gene family, is currently being evaluated for this role in CaP (6). Increased expression of PSMA has been correlated with more aggressive disease (7). CaP-associated expression of PSMA is also being evaluated for imaging of prostate cancer by radiolabeled anti-PSMA monoclonal antibodies (7). Furthermore, promising immunotherapy approaches are being pursued using PSMA peptide (8). Recent gene discovery approaches have led to the identification of several new prostate-specific/abundant genes, such as *NKX3.1* (9), prostase (10), prostate stem cell antigen (11), *TMPRSS2* (12), *STEAP* (13), *PDEF* (14), *PART-1* (15), *HOXB13* (16), *DD3* (17), *PCGEM1* (18), and *PMEPA1* (19), that exhibit diverse characteristics.

In this report, we describe the discovery of a new prostate-specific gene, *PSGR*, a member of the G-protein coupled OR family that maps to chromosome 11p15. The most striking aspects of *PSGR* characterization are its highly prostate tissue-specific expression and its tumor-associated overexpression. The predicted protein sequence of the *PSGR* revealed seven transmembrane-spanning domains with homology to G protein-coupled receptor odorant receptors. Tumor-associated overexpression and the prostate tissue-specific nature of *PSGR*, a G protein-coupled *trans*-membrane receptor, suggest its potential as a novel target for immunotherapeutic strategies of prostate cancer.

Materials and Methods

Identification of *PSGR*. *PSGR* was identified as a prostate tissue-specific cDNA, HPAJ during a search of the expressed sequence tag database at Human Genome Sciences using approaches that previously led to the identification of *NKX3.1* (9).

Received 7/17/00; accepted 10/17/00.

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¹ Supported in part by a grant from the Center for Prostate Disease Research, a program of the Henry M. Jackson Foundation for the Advancement of Military Medicine (Rockville, MD), funded by the United States Army Medical Research and Materiel Command.

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⁴ The abbreviations used are: CaP, carcinoma of prostate; PSA, prostate-specific antigen; GPCR, G protein-coupled receptor; OR, odorant receptor; HK, human kallikrein; PSMA, prostate-specific membrane antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; ORF, open reading frame.

Analysis of Tissue-specific Expression of *PSGR* Using MTN Blots. Multiple tissue Northern blots containing mRNA samples from 23 human tissues and Master dot blots containing mRNA samples from 50 different human tissues (Clontech) were hybridized with the [α - 32 P]dCTP-labeled 513-bp *PSGR* cDNA fragment, which was amplified from a *PSGR* cDNA clone using primers 5'-GCCACCTGTGTGCTTATTGGTATCC-3' (sense) and 5'-GACACAATGAGGATGCGAGAGGACATTG-3' (antisense). As an internal control, *GAPDH* probe was used to hybridize all of the blots.

Prostate Tissue Specimens, Pathological Evaluation, and Microdissection. Matched prostate cancer and normal tissues were derived from radical prostatectomy specimens from 52 prostate cancer patients treated at Walter Reed Army Medical Center (under an institutional review board-approved protocol). All radical prostatectomy patients in this study are enrolled in the Department of Defense Center for Prostate Disease Research Triservice Multicenter Longitudinal Prostate Cancer Database. The genitourinary pathologist (I. A. S.) was present in the operating room to immediately accept the prostate gland from the urologists. If a palpable tumor was present, the surface overlying it was painted with black ink, and a wedge from the center was immediately embedded in Tissue-tek OCT (Miles Inc. Diagnostics Division, Elkhart, IN) and frozen at -70°C . Sextant 14-gauge true cut biopsies were also obtained on each case and processed similarly. Sextant biopsies include apex, mid, and base of the right and left lobes of the prostate performed *ex vivo*. The volume of biopsy specimens was about $1 \times 0.5 \times 0.5 \text{ cm}^3$. Ten- μm frozen sections were prepared and archived at -70°C . One set of slides was stained with H&E and read by the pathologist (I. A. S.) to define tumor cells. The frozen sections on slides were dissected using laser captured microdissection (35 pairs) according to the protocol provided by the manufacturer (Arcturus Engineering, Mountain View, CA) or manual microdissection (17 pairs). Total RNA was prepared from harvested normal and tumor prostate epithelial cells as described previously (20). The expression of *PSGR* was correlated with clinicopathological features of the patients.

RT-PCR Assay. Total RNA (100 ng) was reverse transcribed into cDNA with a RNA PCR kit (Perkin-Elmer, Foster, CA), and one-tenth of the reverse-transcribed product from each sample was used for PCR to amplify *PSGR* and a housekeeping gene, *GAPDH*. The primer sequences for amplification of *PSGR* were the same as those described above. The conditions of PCR for each gene were optimized to analyze the amplified product in the linear range of amplification by adjusting amplification cycles for each set of primers. The optimized PCR condition for amplifying *PSGR* was 36–42 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 30 s. The expression of *GAPDH* was used as an internal control for RNA input (20). Expression of *GAPDH* and *PSGR* was analyzed simultaneously with the same batch of cDNA. Other controls for the RT-PCR assays included PCR amplification of the RNA samples without reverse transcription. Four randomly selected RT-PCR products of the *PSGR* were sequenced to confirm their identity as *PSGR*. The computer-stored images of ethidium bromide-stained agarose gels were analyzed by densitometry of the bands with NIH image processing software. The results of *PSGR* gene expression were presented as relative expression by using the ratio of the intensities of PCR product of *PSGR* to that of *GAPDH*. *PSGR* expression was further quantified as differential expression between tumor (T) and normal (N) tissues as follows: (a) overexpression in tumor tissue ($T > N$), 1+ (1.5–5-fold), 2+ (6–10-fold), 3+ (11–20-fold), and 4+ (>20-fold) increased expression as compared with matched normal tissue; (b) reduced expression in tumor tissue ($T < N$), 1– (1.5–5-fold), 2– (6–10-fold), 3– (11–20-fold), and 4– (>20-fold) decreased expression as compared with matched normal tissue; and (c) no change ($T = N$), the difference in *PSGR* expression between tumor and normal tissue was <1.5-fold. No detectable *PSGR* expression in one of the specimens of tumor/normal pairs was scored as 4+ for increased expression or 4– for decreased expression.

RNA from paired normal and tumor specimens from 8 of the 52 patients was also analyzed by real-time RT-PCR using the 7700 sequence detection system (PE Applied Biosystems, Foster, CA). Real-time RT-PCR was conducted using 10 ng of total RNA from paired normal and tumor samples. PCR primers for *PSGR* were CATGGCCTTTGACCGTTATGT: GCCAATCTGGGCTGTACTGTAT using a FAM-labeled probe (ACCCATGCGC-CATGCTGCA). 18S rRNA was detected as internal control. Reverse transcription reactions were carried out at 48°C with or without Moloney murine leukemia virus reverse transcriptase for 30 min. PCR reactions were performed in 25 μl volumes containing the manufacturer's recommended buffer supple-

mented with 8% glycerol, 0.4% Tween 20, and 0.05% gelatin. Paired quadruplicate samples (one lacking reverse transcription and triplicates with reverse transcription) were heat-denatured for 10 min at 95°C and then amplified using either the 18S or *PSGR* probe primer combinations. Amplification consisted of 40 cycles using a melting (15 s, 95°C) and annealing/extension (60 s, 60°C) step. Results were plotted as average cycle threshold (cT) values for each triplicate sample minus the average triplicate values for 18S rRNA. Differences between tumor and normal samples were calculated using the formula $2^{\exp(cT_{\text{tumor}} - cT_{\text{normal}})}$ and expressed as fold change in expression.

In Situ Hybridization of *PSGR* in Prostate Tissues. A 513-bp PCR fragment (nucleotides 298–810; Fig. 1) was amplified from the cDNA clone of *PSGR* and cloned into a PCR blunt II-TOPO vector (Invitrogen, Carlsbad, CA). Digoxigenin-labeled antisense and sense riboprobes were synthesized using an *in vitro* RNA transcription kit (Boehringer Mannheim, Indianapolis, IN), and a linearized plasmid with *PSGR* gene fragment as templates. The hybridization was performed as described previously (19). The slides were evaluated under an Olympus BX-60 microscope.

Results

***PSGR* Is a New Member of G Protein-coupled Olfactory Receptors.** Analysis of the 1474-bp *PSGR* cDNA sequence (Fig. 1A), previously named as HPRAJ, revealed an ORF of 963 bp nucleotides encoding a 320-amino acid protein with a predicted molecular mass of 35.4 kDa. The *PSGR* ORF revealed intriguing homology (~50% identity and ~70% similarity) to the G protein-coupled odorant receptor family. Specifically, the analysis of *PSGR* with Dnastar software showed the closest similarity of *PSGR* to a human OR gene, HPPH1OR, which was mapped to the β -globin gene cluster on chromosome 11p15.5 (Fig. 1B; Ref. 21). Furthermore, *PSGR* ORF exhibited almost complete identity to a rat cDNA sequence (accession number AF079864) in the NCBI GenBank.⁵ A protein motif search using ProfileScan⁶ indicated the existence of seven trans-membrane domains between amino acid residues 24–293 that are characteristic of GPCRs. Chromosomal mapping assigned *PSGR* to 11p15 (data not shown), one of the regions of GPCR clusters.

Prostate Tissue-specific and Epithelial Cell-restricted Expression of *PSGR*. The distribution of *PSGR* mRNA in normal human tissues was examined by Northern blot analysis. Of the 23 different human tissue mRNAs analyzed, a ~2.7-kb mRNA transcript specifically hybridizing to the *PSGR* cDNA was detected only in prostate tissue (Fig. 2A). Two independent experiments revealed identical results. Further analysis of RNA Master blot containing RNA from 50 different human tissues confirmed the prostate tissue specificity of the *PSGR* gene (data not shown). Among the prostate cancer cell lines analyzed, weak expression of *PSGR* was detected in LNCaP cells by RT-PCR and Northern hybridization (Fig. 2B). *In situ* RNA hybridization analysis of *PSGR* expression in prostate tissues revealed that expression of *PSGR* was predominantly localized to epithelial cells of the prostate gland (Fig. 2C). Therefore, a comprehensive expression analysis of *PSGR* in human prostate tissues revealed prostatic epithelial cell-specific expression of the *PSGR*.

***PSGR* Expression in Prostate Cancer.** Fig. 3 shows a representative semiquantitative RT-PCR analysis of *PSGR* and *GAPDH*. The overall expression pattern of the *PSGR* gene is shown in Table 1. Comparison of *PSGR* expression between normal and tumor tissues revealed overexpression in 62% of tumor specimens (32 of 52 specimens), decreased expression in 11.5% of specimens (6 of 52 specimens), and no change in 27% of specimens (14 of 52 specimens). The cumulative *PSGR* expression level in tumor tissues (0.4350 ± 0.4987) are significantly higher ($P = 0.001$) than that in normal tissues (0.1636 ± 0.2843). Quantitative RT-PCR of eight pairs of tumor and

⁵ Internet address: www.ncbi.nlm.nih.gov.

⁶ Internet address: <http://www.ch.embnet.org/cgi-bin/TMPRED>.

A

CCATGCTGCCTCCGGGCGAGTACCATCCATCTCCACACCCCTGGAAGACACAGTGAAGTTAGCACCACCACCAGGTAATGGCCTTATCAGCTCTGTGCCTGTCTCCAGTCAGGCTGGAATA 120

AGTCTCCTCATATGTGCAAGCTCGGCCCTCCCGTGAATCTAAAGCCTCTCAGCCTTCTGAGTCAGCCTGAAAGGAACAGGCCGAAGTCTGTATGGGCTCTACTGCCAGTGTGACCTC 240

ACCCCTCCAGTCACCCCTCTCAGTCCAGT**ATG**AGTTCCCTGCAACTTCACACATGCCACCTTTGTGCTTATGGTATCCAGGATTAGAGAAAGCCATTTCTGGGTTGGCTTCCCC 360
 M S S C N F T H A T F V L I G I P G L E K A H F W V G F P 29

CTCCTTTCCATGTATGTAGTGGCAATGTTTGGAACTGCATCGTGGTCTTCATCGTAAGGACGGAAACCGCAGCCTGCACGCTCCGATGTACCTCTTTCTCTGCATGCTTGCCAGCCATTGAC 480
 L L S M Y V V A M F G N C I V V F I V R T E R S L H A P M Y L F L C M L A A I D 69

CTGGCCTTATCCACATCCACCATGCCTAAGATCCCTTGGCCCTTTTCTGGTTTGGATTCCCGAGAGATTAGCTTTGAGGCCTGTCTTACCAGATGTCTTTTATTTCATGCCCTCTCAGCCATT 600
 L A L S T S T M P K I L A L F W F D S R E I S F E A C L T Q M F F I H A L S A I 109

GAATCCACCATCCTGCTGGCCATGGCCTTTGACCGTTATGTGGCCATCTGCCACCCACTGGCCATGCTGCAGTGTCAACAATACAGTAACAGCCAGATTGGCATCGTGGCTGTGGTC 720
 E S T I L L A M A F D R Y V A I C H P L R H A A V L N N T V T A Q I G I V A V V 149

CGCGGATCCCTCTTTTTTCCCACTGCCTCTGTGTGATCAAGCGGCTGGCCTTCTGCCACTCCAATGTCTCTCGCACTCTTATTGTGTCCACCAGGATGTAATGAAGTTGGCCTATGCA 840
 R G S L F F F P L P L L I K R L A F C H S N V L S H S Y C V H Q D V M K L A Y A 189

GACACTTGGCCAAATGTGGTATATGGTCTTACTGCCATTCTGCTGGTTCATGGGCGTGGACGTAATGTTTCATCTCCTTGTCTCTTTTCTGATAATACGAACGGTTCTGCAACTGCCTTCC 960
 D T L P N V V Y G L T A I L L V M G V D V M F I S L S Y F L I I R T V L Q L P S 229

AAGTCAGAGCGGGCCAGGCGCTTGGAACTGTGTGCACACATGGTGTGGTACTCGCCTTCTATGTGCCACTTATGGCCTCTCAGTTGTACACCCTTTGGAAACAGCCTTCATCCC 1080
 K S E R A K A F G T C V S H I G V V L A F Y V P L I G L S V V H R F G N S L H P 269

ATTGTGCGTGTTCATGGGTGACATCTACCTGCTGCTGCCTCCTGTCAATCCCATCATCTATGGTGCCAAAACCAACAGATCAGAACACGGGTGCTGGCTATGTTCAAGATCAGC 1200
 I V R V V M G D I Y L L L P P V I N P I I Y G A K T K Q I R T R V L A M F K I S 309

TGTGACAAGGACTTGCAGGCTGTGGGAGGCAAG**TGA**CCCTTAACACTACACTTCTCCTTATCTTTATTGGCTTGATAAACATAATTATTCTAACACTAGCTTATTCCAGTTGCCATA 1320
 C D K D L Q A V G G K * 320

AGCACATCAGTACTTTTCTCTGGCTGGAATAGTAACTAAAGTATGGTACATCTACCTAAAGGACTATTATGTGGAATAATACATACTAATGAAGTATTACATGATTTAAAGACTACAAT 1440

AAAACCAACATGCTTATAACATTAATAAAAAA 1474

B

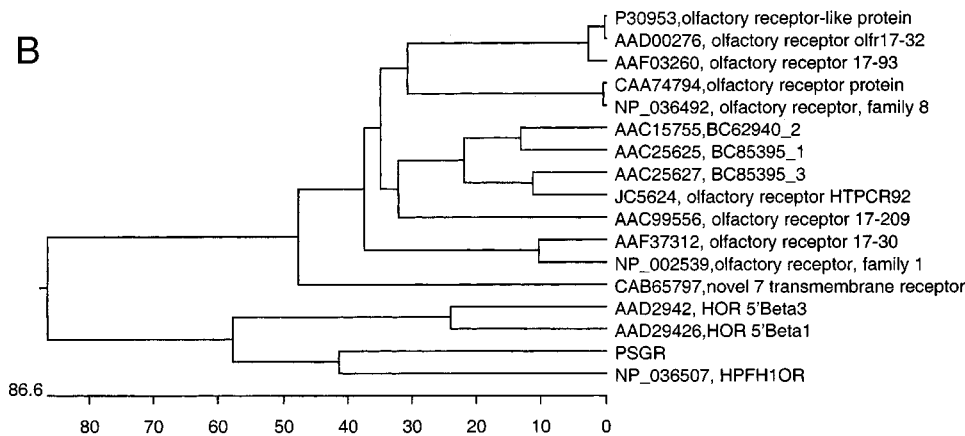


Fig. 1. Characterization of *PSGR* cDNA sequence. A, nucleotide and predicted amino acid sequence of *PSGR*. The potential initiation methionine codon and the translation stop codon are indicated in bold. The transmembrane domains are underlined. B, multiple alignments of the predicted protein sequence of *PSGR* and other human GPCRs shown as a phylogenetic tree. The right part shows the GenBank accession numbers and the name of those genes (the accession number of *PSGR* is AF311306).

normal tissue analyzed by Taqman procedure revealed tumor-associated overexpression in 7 tumors consistent with the semiquantitative RT-PCR (data not shown). As a complement to RT-PCR, *PSGR* expression was also analyzed by *in situ* RNA hybridization in selected specimens. Of the 10 cases analyzed, 7 showed significantly elevated expression of *PSGR* in malignant prostate epithelial cells as compared with adjacent benign cells (Fig. 2C). Thus, using three different methods, we have confirmed overexpression of *PSGR* in prostate cancer. Preliminary analysis of *PSGR* expression did not show a significant correlation with specific clinicopathological features (such

as pathologic and clinical stage) (data not shown). A larger study of whole mount prostate specimens is in progress for further investigation of this issue.

Discussion

Recent gene discovery efforts in prostate cancer have been instrumental in the identification numerous new prostate-specific genes. The biological and biochemical characterization of these genes has a significant impact in understanding the role of these genes in prostate biology. Furthermore, potential utilities of prostate-specific molecules

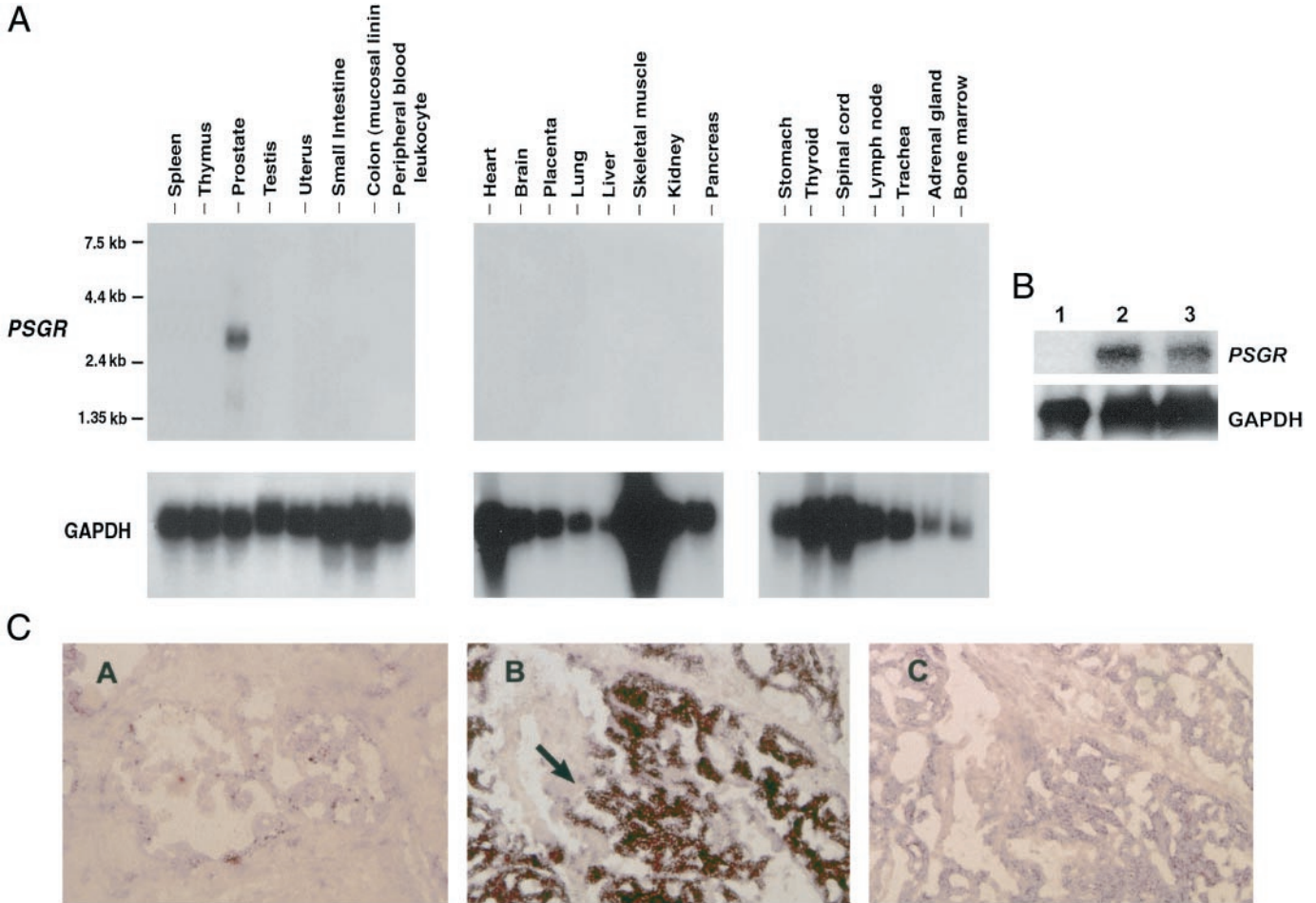


Fig. 2. PSGR expression by Northern hybridization and *in situ* hybridization analysis. **A**, multiple tissue Northern blots were hybridized with an [α - 32 P]dCTP-labeled *PSGR* cDNA fragment. A 2.7-kb mRNA transcript specifically hybridizing to *PSGR* cDNA was detected only in prostate tissue. **B**, *PSGR* expression in prostate cancer cell lines. Eight μ g of polyadenylated RNA were used to make the Northern blot. The blot was hybridized with [α - 32 P]dCTP-labeled *PSGR* probe. The samples are RNA from PC3 cells (*Lane 1*), LNCaP cells cultured in androgen-free medium for 5 days and then stimulated with R1881 at 10^{-8} M for 24 h (*Lane 2*), and LNCaP cells processed as described for *lane 2* but without R1881 stimulation (*Lane 3*). **C**, *in situ* hybridization analysis. **A** and **B** show the hybridization signal with *PSGR* antisense riboprobe in benign and malignant prostate tissues from the same patient. **C** shows a similarly processed hybridization with sense *PSGR* riboprobe. The arrow shows the restricted expression of *PSGR* in glandular epithelial cells.

as biomarkers of prostate cancer onset/progression and as a cancer vaccine target are obvious in a clinical setting. PSA (or HK3) as a biomarker for prostate cancer is a success story in human cancer, despite certain limitations. The identification of additional biomarkers that are not only prostate specific but are also overexpressed in cancer will be of special interest. Genes such as *TMPRSS2*, *STEAP*, *PDEF*, *DD3*, and *PCGEM1* (12–14, 17–18) represent efforts in this direction. Here we report novel observations on the identification of a putative GPCR with a remarkable prostate tissue specificity showing tumor-specific overexpression in a large percentage of tumors. The homology of *PSGR* to the G protein-coupled odorant receptor genes is

Table 1 *PSGR* expression status in prostate cancer

PSGR expression in 52 matched prostatic cancer (T) and normal tissues (N) was analyzed by RT-PCR. The results were represented as differential expression in T and N as described in "Materials and Methods." T > N, overexpression in tumor tissue, graded as 1+ (1.5–5-fold), 2+ (6–10-fold), 3+ (11–20-fold), and 4+ (>20-fold) increased expression as compared with matched normal tissue; T < N, reduced expression in tumor tissue, graded as 1– (1.5–5-fold), 2– (6–10-fold), 3– (11–20-fold), and 4– (>20-fold) decreased expression as compared with matched normal tissue; and T = N, similar expression in tumor and normal tissue, refers to the difference of *PSGR* expression between tumor and normal tissues as <1.5-fold (graded as 0).

Group	No. of patients/group (%)	Expression score (no. of specimens)
T > N	32 (61.5)	1+ (13) 2+ (3) 3+ (5) 4+ (11)
T < N	6 (11.5)	1– (2) 4– (4)
T = N	14 (26.9)	0 (14)

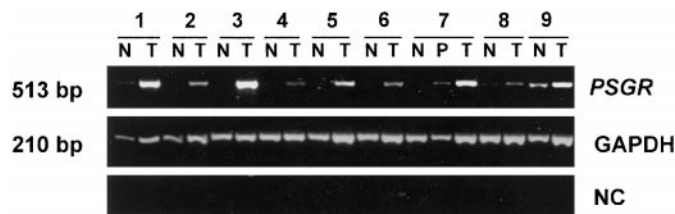


Fig. 3. *PSGR* expression in primary prostate cancer specimens by RT-PCR. Representative RT-PCR products of tumor and adjacent normal tissues were analyzed on agarose gel. Lanes 1–9 represent PCR results of paired normal and tumor tissues from the same patient. NC, no reverse transcription (control); N, normal prostate tissue; P, prostatic intraepithelial neoplasia; T, prostate tumor tissue.

particularly intriguing because odorant receptors are almost exclusively localized in nasal epithelium. It is important to note that GPCR genes belong to the largest "gene family" in the human genome, and it has been argued that odorant receptors have emerged directly for GPCRs and that any GPCR has the potential to become an OR at a given point in evolution (22). In addition to nasal epithelium, odorant receptor genes have been detected in testis, and their functional

relevance remains unclear (23). This is the first documentation of the prostate-specific expression of a gene with OR homology. Therefore, pending experimental validation, *PSGR* should be considered a GPCR and not an OR. Future studies are required to determine the biological significance of the OR-like genes in prostate and testes. *PSGR* expression also appears to be tissue context specific because we were unable to detect *PSGR* expression in a number of cultured primary prostate cancers cells or immortalized cell lines and established metastatic prostate cancer cell lines. However, weak expression of *PSGR* was detected only in LNCaP prostate cancer cells. In this regard, it is important to mention that expression of several prostate-specific genes including PSA is often lost during cell culture condition. Another important finding relates to a highly significant overexpression of *PSGR* in prostate cancer cells. Of note also is the restricted expression of *PSGR* in epithelial cells of the prostate gland. In summary, we have demonstrated prostatic epithelial cell-restricted expression of *PSGR*, and tumor cells exhibit significantly increased expression of this putative seven-transmembrane GPCR. However, it is not yet clear how *PSGR* overexpression may play a role in the process of tumorigenesis. GPCR may play important roles in cell signaling and cell proliferation (24). Therefore, future experiments will focus on the biological function of *PSGR* in tumorigenesis of CaP. Membrane localization of the *PSGR* makes *PSGR* protein an attractive target for immunotherapy approaches and antibody-based imaging of metastatic prostate cancer. It also will be very important to determine the signals transduced by *PSGR* in the prostate gland, and future studies will address these issues.

Acknowledgments

We thank Roger Connelly for performing the statistical analysis.

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Cancer Res 2000;60:6568-6572.

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