Method of identifying and treating invasive carcinomas (CIP)

3-8-2005

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Recommended Citation

Chai, Xinqing; Chao, Julie; Chao, Lee; and Chen, Limei, "Method of identifying and treating invasive carcinomas (CIP)" (2005). UCF Patents. Paper 355.
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METHOD OF IDENTIFYING AND TREATING INVASIVE CARCINOMAS

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Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 90 days.

Filed: Sep. 5, 2002

Related U.S. Application Data

Continuation application No. 09/755,811, filed on Jan. 5, 2001, now Pat. No. 6,569,684.

Provisional application No. 60/174,801, filed on Jan. 6, 2000.

Abstract

Prostasin protein has been found to be a useful marker for determination of the invasiveness of and as a means to treat human carcinomas. Using RT-PCR and western blot analyses, prostasin protein and mRNA expression were found in normal human prostate epithelial cells and the human prostate cancer cell line LNCaP, but not in the highly invasive human prostate cancer cell lines DU-145 and PC-3. Immunohistochemistry studies of human prostate cancer specimens revealed a down-regulation of prostasin in high-grade tumors. Using RT-PCR and western blot analyses, prostasin protein and mRNA expression were found in a non-invasive human breast cancer cell line, MCF-7, while invasive human breast cancer cell lines MDA-MB-231 and MDA-MB-435s were found not to express either the prostasin protein or the mRNA. A non-invasive human breast cancer cell line, MDA-MB-435s, was shown to express prostasin mRNA to detect prostate cells in blood of cancer patients. Clinical and Chemical Laboratory Medicine 37 (SS): S119, 1999.

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(57) ABSTRACT
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### Fig. 1

**Human Prostate Gland 20**

<table>
<thead>
<tr>
<th>Normal or Non-Invasive Cancer</th>
<th>YES! 22</th>
<th>NO! 24 or Reduced 26</th>
</tr>
</thead>
</table>

**Prostasin-Serine Protease 14**

<table>
<thead>
<tr>
<th>Normal</th>
<th>NO? 12</th>
<th>YES? 16</th>
</tr>
</thead>
</table>

**Human Blood 19**

### Fig. 2

**Human Mammary Gland 200**

<table>
<thead>
<tr>
<th>Non-Invasive Cancer</th>
<th>YES! 220</th>
<th>NO! 240 or Reduced 260</th>
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</table>

**Prostasin Serine Protease 140**

<table>
<thead>
<tr>
<th>Normal</th>
<th>NO? 120</th>
<th>YES? 160</th>
</tr>
</thead>
</table>

**Human Blood 160**
Fig. 3

Prostasin 40 kDa
Prostasin 232 bp
β-Actin 556 bp

CC-2595  LNCaP  DU-145  PC-3
Fig. 6

Prostate

1,275 bp →
951 bp →
CC-2555 LNCaP DU-145 PC-3

Breast

1,275 bp →
951 bp →
MCF-7 MDA-453 MDA-231 MDA-435
Fig. 7

Vector Prostasin

40 kDa

DU-145 PC-3

Relative Invasiveness (percentage)

Fig. 8

Vector Prostasin

40 kDa

MDA-MB-231 MDA-MB-435c

Relative Invasiveness (percentage)
Figure 9
METHOD OF IDENTIFYING AND TREATING INVASIVE CARCINOMAS

This invention relates to prostasin and its use in the diagnosis and treatment of prostate and breast cancers, and is a Continuation-In-Part (CIP) of application Ser. No. 09/755,811 filed Jan. 5, 2001 now U.S. Pat. No. 6,569,684 and claims the priority to U.S. Provisional application Ser. No. 60/174,801 filed Jan. 6, 2000 and was supported in part by Department of Defense Prostate Cancer Research Grant DAMD17-98-1-8590, and in part by grants to principal investigator K. X. CHAI from the Florida Hospital Gala Endowed Program for Oncologic Research.

BACKGROUND AND PRIOR ART

For men in the U.S. prostate cancer is the most commonly diagnosed cancer, and the second leading cause of cancer-related death (Greenlee R T; Murray F; Bolden S; Wingo P A. Cancer statistics, 1999. Ca: a Cancer Journal for Clinicians 2000;50:7-33). Prostate cancers originate as localized lesions; some of these localized lesions will progress to become invasive, migratory and metastatic. Our current understanding of the mechanisms of the prostate cancer invasion process, however, is poor. Our ability to predict the acquisition of invasive potentials by a prostate cancer is limited. The mechanisms leading to the development of a prostate cancer are complex. Currently, it is believed to be the result of multiple transformation steps from normal prostate glandular cells (Carter H B; Piantadosi S; Isaacs J T. Clinical evidence for and implications of the multistep development of prostate cancer. Journal of Urology. 143(4):742–6, 1990). The initial steps result in what are described as prostatic interepithelial neoplastic (PIN) lesions (Isaacs J T. Molecular markers for prostate cancer metastasis. Developing diagnostic methods for predicting the aggressiveness of prostate cancer. [Review] [92 refs] American Journal of Pathology. 150(5):1511–21, 1997). These PIN lesions may then typically have three different fates based on an assessment of their impact to the patient. The PIN lesions can remain as such, not producing histologically detectable prostate cancer, or further transform into histologically detectable prostate cancer. Most of the histologically detectable prostate cancers will be asymptomatic in the patient and remain non-manifest clinically as many are discovered post-mortem (Carter H; Coffey D. Prostate Cancer: the magnitude of the problem in the United States. In: A Multidisciplinary Analysis of Controversies in the Management of Prostate Cancer. (Eds. Coffey D; Resnick M; Door R. et al.), pp1–9, Plenum Press, 1988; Carter H B; Piantadosi S; Isaacs J T. Clinical evidence for implications of the multistep development of prostate cancer. Journal of Urology. 143(4):742–6, 1990; Scardino P T; Weaver R; Hudson M A. Early detection of prostate cancer. [Review] [102 refs] Human Pathology. 23(3):211–22, 1999). Prostate cancers are diagnosed clinically by an estimate of size and location using the TNM staging system (Denis L J. Staging and prognosis of prostate cancer. European Urology. 24 Suppl 2:13–8, 1993), and by pathological staging based on an examination of the histology of the removed prostate via either biopsy or prostatectomy using a system by D. F. Gleason, (Gleason D F. Classification of prostatic carcinomas. Cancer Chemotherapy Reports—Part 1. 50(3):125–8, 1966). About 50% of prostate cancer cases receiving treatment are diagnosed clinically as advanced, or, non-organ-confined (Scardino P T; Weaver R; Hudson M A. Early detection of prostate cancer. [Review] [102 refs] Human Pathology. 25(3):211–22, 1992), for which no effective treatment exists (Yagoda A. Petrylak D. Cytotoxic chemotherapy for advanced hormone-resistant prostate cancer. [Review] [63 refs] Cancer. 71(3 Suppl): 1088–109, 1993 and Petrylak D. Cytotoxic chemotherapy for advanced hormone-resistant prostate cancer. Journal of Urology. 143(4):742–6, 1990). Of the remaining 50% cases, ½ (~50,000) are diagnosed as organ-confined but micrometastasis may be present. The final group of patients (~100,000) have truly organ-confined prostate cancer and can be cured by radical prostatectomy (Sgrignoli A R; Walsh P C; Steinberg G D; Steiner M S; Epstein J. Prognostic factors in men with stage D 1 prostate cancer: identification of patients less likely to have prolonged survival after radical prostatectomy [see comments]. Journal of Urology. 152(4):1077–81, 1994; Zincke H; Oesterling J E; Blute M L; Bergstralh E J; Myers R P; Barrett D M. long term (15 years) results after radical prostatectomy for clinically localized (stageT2c or lower) prostate cancer [see comments]. Journal of Urology.152(5 Pt 2): 1850–7, 1994) or left untreated (watchful waiting) without the risk of life-threatening or life-altering. For the patients with non-organ-confined prostate cancers (discovered via either biopsy or surgery), undergoing systemic treatment early is essential to the management of their cancer (Yagoda A. Petrylak D. Cytotoxic chemotherapy for 108-109, 1993). Consequently, it will be ideal, both medically and economically, if one could precisely predict upon early pathological examination of the tumor, which group of patients will have truly organ-confined disease versus which group will have invasive prostate cancer.

Clinical staging of prostate cancer generally depends on the results of three tests that are performed in the following order: a PSA (prostate-specific antigen) blood test as a screening method; DRE (digital rectal examination) for an initial indication of palpable disease; and, a biopsy to obtain samples for histological examination. Prostate cancers, removed either via biopsy or surgery, are graded histologically by the system of Gleason (Gleason D F. Classification of prostatic carcinomas. Cancer Chemotherapy Reports—Part 1. 50(3):125–8, 1966), which is an evaluation of how aggressive and how poorly-differentiated the prostate cancers are. The aggressiveness of prostate tumors: of low Gleason scores (<5) is limited; of high Gleason scores (8–10) are highly aggressive; but, for the intermediate Gleason-score (5-7) prostate cancers (76% of prostate tumors), the accuracy of predicting their aggressiveness is poor (Gleason D F; Mellinger G T. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. Journal of Urology. 111(1): 58–64, 1974). Thus, the ability to accurately determine the aggressiveness of these intermediate Gleason-score prostate tumors has remained as a practical challenge to, and a primary goal for, prostate cancer research (Isaacs J T. Molecular markers for prostate cancer metastasis. Developing diagnostic methods for predicting the aggressiveness of prostate cancer. [Review] [92 refs] American Journal of Pathology. 150(5):1511–21, 1997). Especially with regard to the number of patients (150,000) facing a decision of whether to undergo systemic treatment, the most urgent demand in prostate cancer care is the development of methods to enhance our ability to accurately predict the aggressiveness of the tumors with Gleason scores of 5–7. It is now commonly believed that cancers occur via multiple transformation steps by accumulating mutations in three classes of genes: proto-oncogenes (Park M. Oncogenes. In The Genetic Basis of Human Cancer (Eds. Vogelstein B and Kinzler K W), pp205–28, McGraw-Hill Health Profession Divisions, 1998); tumor-suppressor genes (Knuttila S; Aalto Y; Bjorkqvist A M; El-Rifai W; Hemmer

to cancer is usually regarded of a beneficial effect for the presence of these molecules (Kennedy AR. Chemopreventive agents: protease inhibitors. Pharmacol Therapeut 78:167–209, 1998). Recently, however, the picture of a new paradigm is beginning to emerge for several serine proteases in breast, prostate, and testicular cancers. A “normal epithelial cell specific-1” (NES1) serine protease was found to be down-regulated in breast and prostate cancers, and it functions as a tumor suppressor (Goyal J. Smith K M. Cowan J M. Wazer D E. Lee S W. Band V. The role for NES1 serine protease as a novel tumor suppressor. Cancer Res 58:4782–4786, 1998). A prostate-specific serine protease, testisin (Nelson P S. Gan L. Ferguson C. Moss P. Gelinas R. Hood L. Wang K. Molecular cloning and characterization of testisin, an androgen-regulated serine protease with prostate-restricted expression. Proceedings of the National Academy of Sciences of the United States of America. 96(6):3114–9, 1999), was shown to be expressed in normal prostate but not in prostate cancer cell lines DU-145 and PC-3. The expression of a testisin-specific serine protease, testisin, was shown to be lost in testicular cancer through either a loss of gene (Hooper J D. Nicol D L. Dickinson J L. Eyre H J. Scarman A L. Nормyle J F. Stuttgen M A. Douglas M L. Loveland K A. Sutherland G R. Antalis T M. Testisin, a new human serine protease expressed by premeiotic testicular germ cells and lost in testicular germ cell tumors. Cancer Research. 59(13):3199–205, 1999) or methylation in the promoter (Boucaut K. Douglas M. Clements J. Antalis T. The serine protease testisin may act as a tumor and/or growth suppressor in the testis and may be regulated by DNA methylation. Cancer Genetics and Tumor Suppressor Genes, Cold Spring Harbor Laboratory, 2000). Further, transfection of human testicular cancer cells with a testisin cDNA reduced the tumor growth of xenografts of these cells in nude mice, suggesting a tumor suppressor function for testisin (Boucaut K. Douglas M. Clements J. Antalis T. The serine protease testisin may act as a tumor and/or growth suppressor in the testis and may be regulated by DNA methylation. Cancer Genetics and Tumor Suppressor Genes, Cold Spring Harbor Laboratory, 2000). The testisin serine protease is potentially membrane-bound as suggested by its structure and confirmed by immunohistochemistry gene (Hooper J D. Nicol D L. Dickinson J L. Eyre H J. Scarman A L. Normyle J F. Stuttgen M A. Douglas M L. Loveland K A. Sutherland G R. Antalis T M. Testisin, a new human serine protease expressed by premeiotic testicular germ cells and lost in testicular germ cell tumors. Cancer Research. 59(13):3199–205, 1999). At the amino acid level, prostasin is similar to plasma kallikrein, coagulation factor XI, hepsin, plasminogen, acrosin, prostate, and, in particular, testisin (sharing 44% sequence identity) [Nelson PS. Gan L. Ferguson C. Moss P. Gelinas R. Hood L. Wang K. Molecular cloning and characterization of testisin, an androgen-regulated serine protease with prostate-restricted expression. Proceedings of the National Academy of Sciences of the United States of America. 96(6):3114–9, 1999; Horisberger J D. Rossier B C. An epithelial serine protease activates the amiloride-sensitive sodium channel. Nature. 389(6651):607–10, 1997]. Prostasin is encoded by a single-copied gene, which is located on human chromosome 16pl 35. Those with low risks of developing invasive breast cancer will have a higher confidence in choosing breast-conserving options, while those at high risks will ponder more aggressive options for patients with DCIS could then be stratified. Those with low risks of developing invasive breast cancer will have a higher confidence in choosing breast-conserving options, while those at high risks will ponder more aggressive options with the necessary follow-up treatments. Similarly, those males with low risks of developing invasive prostate cancer will have a higher confidence in choosing prostate-conserving options, while those at high risks will ponder more aggressive options with the necessary follow-up treatments.

SUMMARY OF THE INVENTION

The first objective of the present invention is to reduce deficiencies in the prior art with specific regard to different-
tial diagnosis of invasive prostate and breast cancers and to treatment of invasive and metastatic prostate and breast cancers.

The second objective of the present invention is to provide a new marker for prostate and breast cancer.

The third objective of the invention is to provide as a drug to patients with carcinoma of the prostate via delivery of a functional prostasin gene.

The fourth objective of the invention is to provide as a drug to patients with carcinoma of the prostate via delivery of a functional prostasin cDNA.

The fifth objective of the invention is to provide as a drug to patients with carcinoma of the breast via delivery of a functional prostasin gene.

The sixth objective of the invention is to provide as a drug to patients with carcinoma of the breast via delivery of a functional prostasin gene.

This invention identifies prostasin serine protease as a potential marker, and as a tumor invasion suppressor for prostate and breast cancers and thus provides methods (a), (b), and (c) of determining invasiveness levels of human carcinomas:

(a) using prostasin protein levels, comprising the steps of: sampling a human carcinoma tissue; determining prostasin protein levels in the human carcinoma tissue; preferably by applying an immunological reagent-antibody to said tissue wherein the reagent-antibody becomes bound to prostasin protein in said tissue, and determining invasiveness of the human carcinoma tissue based on the prostasin protein levels.

(b) using prostasin mRNA levels, comprising the steps of: sampling a human carcinoma tissue; determining prostasin mRNA levels in the human carcinoma tissue preferably by applying prostasin-specific anti-sense RNA probes in an in situ hybridization to determine the prostasin mRNA levels in the separated human carcinoma tissue the determination of the prostasin mRNA levels in the separated human carcinoma sample tissue; to make possible and determining invasiveness of the human carcinoma tissue based on the prostasin mRNA levels.

(c) using prostasin gene promoter DNA methylation levels, comprising the steps of: sampling a human carcinoma tissue; determining prostasin gene promoter DNA methylation levels in the human carcinoma tissue, preferably by applying prostasin-promoter-specific oligonucleotide primers in a PCR to determine the prostasin gene promoter DNA methylation levels in the sampled human carcinoma tissue and determining invasiveness of the human carcinoma tissue based on the prostasin gene promoter DNA methylation levels, as well as a method of treating invasive human carcinomas comprising the steps of: incorporating human prostasin nucleic acid into a selected gene delivery vector nucleic acid to form a recombinant nucleic acid; preferably wherein the nucleic acid includes a gene or cDNA delivering the recombinant nucleic acid into a human carcinoma; and reducing invasiveness of the human carcinoma.

Further objects and advantages of this invention will be apparent from the following detailed description of a presently preferred embodiment, which is illustrated schematically in the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURE

FIG. 1 is a diagrammatic representation of the analysis of prostasin indicative of the absence or existence, or of the invasiveness of human prostate carcinoma.

FIG. 2 is a diagrammatic representation of the analysis of prostasin indicative of the absence or existence, or of the invasiveness of human mammary carcinoma.

FIG. 3 shows human prostasin expression in prostate epithelial cells.

FIG. 4A shows immunohistochemical detection of prostasin protein in benign human prostate tissues.

FIG. 4B is an enlarged view of the boxed region of FIG. 4A.

FIG. 4C shows immunohistochemistry of benign human prostate tissues with prostasin antibody omitted in the procedures, no epithelial cells displayed any staining.

FIG. 4D is an enlarged view of the boxed region of FIG. 4C.

FIG. 4E shows immunohistochemistry of human prostate tumor with prostasin antibody omitted in the procedures, no epithelial cells displayed any staining.

FIG. 4F shows immunohistochemical detection of prostasin protein in benign human prostate tissue surrounded by prostate carcinomas.

FIG. 4G shows immunohistochemical detection of prostasin protein in Gleason grade 2 prostate tumor.

FIG. 4H is an enlarged view of the boxed region of FIG. 4G.

FIG. 4I shows immunohistochemical detection of prostasin protein in Gleason grade 3 prostate tumor.

FIG. 4J is an enlarged view of the boxed region of FIG. 4I.

FIG. 4K shows immunohistochemical detection of prostasin protein in Gleason grade 4 prostate tumor.

FIG. 4L is an enlarged view of the boxed region of FIG. 4K.

FIG. 5 shows human prostasin expression in human breast cancer cell lines.

FIG. 6 shows promoter hypermethylation of the human prostasin gene in human prostate and breast cancer cell lines.

FIG. 7 shows prostasin protein expression and in vitro invasive properties of the DU-145 and the PC-3 transfectants.

FIG. 8 shows prostasin protein expression and in vitro invasive properties of the MDA-MB-231 and the MDA-MB-435s transfectants.

FIG. 9a is a schematic illustration of the promoter-exon region of the prostasin gene.

FIG. 9b shows a genomic Southern blot analysis of DNA from NHMEC and human breast cancer cell lines.

FIG. 9c shows a genomic Southern blot analysis of MDA-MB-231 and MDA-MB-435s cells following demethylation.

FIG. 10 shows DNA sequence and the deduced amino acid sequence of the PRSS8 (prostasin) gene.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Before explaining the disclosed embodiment of the present invention in detail it is to be understood that the invention is not limited in its application to the details of the particular arrangement shown since the invention is capable of other embodiments. Also, the terminology used herein is for the purpose of description and not of limitation.

Using RT-PCR and western blot analyses, prostasin protein and mRNA expression were found in normal human prostate epithelial cells and the human prostate cancer cell
line LNCaP, but discovered not present in the highly invasive human prostate cancer cell lines DU-145 and PC-3. Immunohistochemistry studies of human prostate cancer specimens revealed a down-regulation of prostasin in high-grade tumors.

Using RT-PCR and western blot analyses, prostasin protein and mRNA expression were found in a non-invasive human breast cancer cell line, MCF-7, while invasive human breast cancer cell lines MDA-MB-231 and MDA-MB-435s were also discovered not to express either the prostasin protein or the mRNA. A non-invasive human breast cancer cell line, MDA-MB-453, was shown to express prostasin mRNA but not prostasin protein. Examination of the prostasin gene promoter in the human prostate and breast cancer cell lines by Southern blot analysis revealed heterogeneous methylation of the promoter in DU-145, PC-3 and MDA-MB-453 cells, and homogeneous methylation of the promoter in MDA-MB-231 and MDA-MB-435s cells. The prostasin gene promoter in normal human prostate epithelial cells, the LNCaP and the prostasin gene promoter in the human prostate and breast cancer cell lines by Southern blot analysis revealed heterogeneous methylation of the promoter in DU-145, PC-3 and MDA-MB-453 cells, and homogeneous methylation of the promoter in MDA-MB-231 and MDA-MB-435s cells. The prostasin gene promoter in normal human prostate epithelial cells, the LNCaP and the MCF-7 cells was shown to be unmethylated. Transfection of DU-145 and PC-3 cells with a full-length human prostasin cDNA restored prostasin expression and reduced the in vitro invasiveness by 68% and 42%, respectively. Transfection of MDA-MB-231 and MDA-MB-435s cells with a full-length human prostasin cDNA restored prostasin expression and reduced the in vitro invasiveness by 50% for either cell line. Cell proliferation was unaffected by re-expression of prostasin. Our data indicate that prostasin is implicated in normal prostate biology and its down-regulation in prostate cancer, and its absence in invasive prostate and breast cancer cell lines indicates increased invasiveness. Our results also indicate that delivering a functional human prostasin gene to invasive prostate and breast cancers can reduce the invasiveness.

For a facile understanding of the invention embodied herein, reference should now be made to FIGS. 1 and 2. FIG. 1 sets forth a schematic for determining if a male has prostate cancer. Human blood 10 is taken from the male and analyzed for the presence of prostasin serine protease 14. If NO 12 prostasin is found, there is probably little or no cancer. If there is a presence YES 16 of prostasin serine protease 14, there is prostate cancer. If a biopsy of the prostate gland 20 is analyzed for the presence of a normal amount YES 22 of prostasin serine protease 14, there is no cancer or there is non-invasive cancer. If there is NO 24 or Reduced 26 amount of prostasin serine protease 14, there is invasive cancer.

FIG. 2 sets forth a schematic for determining if a female has breast cancer. Human blood 100 is taken from the female and analyzed for the presence of prostasin serine protease 140. If NO 120 prostasin is found, there is prospectively no cancer. If there is a presence YES 160 of prostasin serine protease 140, there is breast cancer. If a biopsy of the breast 200 is analyzed for the presence of YES 220 of prostasin serine protease 140, there is no invasive cancer but may be non-invasive cancer. If there is NO 240 or Reduced 260 amount of prostasin serine protease 140, there is invasive cancer.

The levels of prostasin protein in the epithelial cells of the human prostate gland can be used as a diagnostic marker for the potential invasiveness of prostate tumors. The supporting evidence came from our findings that two invasive human prostate cancer cell lines DU-145 and PC-3 do not express prostasin while non-prostate epithelial cells and a non-invasive prostate cancer cell line LNCaP express both the prostasin mRNA and protein.

Refer now to FIG. 3 which shows human prostasin expression in prostate epithelial cells. By means of western blot analysis (upper panel), prostasin (as a 40-kDa band) was detected in normal human prostate epithelial cells (CC-2555) and the LNCaP cells, but not in the DU-145 or PC-3 cells. An equal amount of total protein (100 µg) was loaded for each sample. At the mRNA level, human prostasin mRNA (via a 232-bp amplified DNA band) was detected in normal prostate epithelial cells (CC-2555) and the LNCaP cells, but not in the DU145 or PC-3 cells as analyzed by RT-PCR, Southern blot hybridization (middle panel). Co-amplification of a 556-bp human β-actin message (as shown in the gel photograph in the lower panel) confirmed the quality and the quantity of the RNA applied in each RT-PCR.

Expression of prostasin protein is reduced in high-grade human prostate tumor. Prostatectomy specimens from 39 patients (128 sections) were subjected to immunohistochemistry using a prostasin-specific antibody. Overall, in non-tumor or benign prostate epithelia, 89.0% of the examined areas demonstrated positive staining for prostasin protein and 11.0% were considered negative (based on the scoring system used for HercepTest™, DAKO Corporation, Carpinteria, CA). In all tumor specimens that were examined, prostasin was detected in 93.3% of the low Gleason grade areas (≤ grade 2), 44.4% of Gleason grade 3 areas, 21.1% of Gleason grade 4 areas, but not in Gleason grade 5 areas (data summarized in Table 1). The mean prostasin immunostaining score was found significantly decreased in high-grade prostate tumors as compared to non-tumor areas (ANOVA, p<0.0001).

Representative staining images of non-tumor (benign) areas and prostate tumor areas are shown in FIGS. 4a-4f, which provides immunohistochemical detection of prostasin protein in tissues. Paraffin-embedded human prostate sections were stained for prostasin protein expression evaluation using a specific antibody as described (Yu JX. Chao L. Chao J. Prostasin is a novel human serine proteinase from seminal fluid. Purification, tissue distribution, and localization in prostate gland. Journal of Biological Chemistry. 269(29):18843-8, 1994). Prostasin positive staining (brown color) was detected in the cytoplasm and apical membrane in non-tumor or benign epithelial cells.

The prostasin protein was detected in the cytoplasm and on the plasma membrane (apical) of benign epithelial cells lining the secretory lumen as well as in the secretion inside the lumen (FIGS. 4A and 4B, score 3, or ++), confirming the results of (Yu JX. Chao L. Chao J. Prostasin is a novel human serine proteinase from seminal fluid. Purification, tissue distribution, and localization in prostate gland. Journal of Biological Chemistry. 269(29):18843-8, 1994). When a pre-immune rabbit serum was used in place of the prostasin antiserum, no staining was observed in either the non-tumor epithelia (FIGS. 4C and 4D) or tumor epithelia (FIG. 4E). Tumor epithelia displayed various degrees of prostasin immunostaining as shown in FIGS. 4F-4L. In Gleason grade 1-2 tumors, moderate prostasin staining is seen in the cytoplasm and on the plasma membrane of some epithelial cells, as well as in the secretion in the lumen (FIGS. 4G and 4H, score 2, or ++). In Gleason grade 3 tumors, a lesser number of epithelial cells displayed the moderate level
prostasin staining (FIGS. 4I and 4J). In Gleason grade 4 tumors, most epithelial cells did not show any prostasin staining, while some prostasin staining can be seen in rare, sporadic tumor cells (FIGS. 4K and 4L, as indicated by the arrow, score 0). Genetically, prostate tumors are heterogeneous and multi-focal in nature, in that one patient’s gross-anatomy tumor comes from multiple initial lesions which are caused by different initial transformation events and progress to different stages by different ensuing transformations (Isaacs J T. Bova GS. Prostate Cancer. In The Genetic Basis of Human Cancer (Eds. Vogelstein B and Kinzler KW), pp653–60, McGraw-Hill Health Professions Division, 1998). The Gleason grading, when used as a percentage of each cancer occupied by Gleason grade 3/4 areas, is independently associated with prostate cancer progression (Stamey T A, McNeal J E, Yemoto C M, Sigal B M, Johnstone I M. Biological determinants of cancer progression in prostate cancer. JAMA 281:1395–400, 1999). We found a significant decrease of prostasin expression in the high-grade, i.e., the more progressively transformed tumors.

As earlier indicated, FIG. 5 shows human prostasin expression in human breast cancer cell lines. By means of western blot analysis (upper panel), prostasin (as a 40-kDa band) was detected in MCF-7 cells, but not in MDA-MB453, MDA-MB-231, or MDA-MB-435s cells. An equal amount of total protein (100 μg) was loaded for each sample. At the mRNA level, human prostasin mRNA (via a 232-bp amplified DNA band) was detected in MCF-7 and MDA-MB453 cells, but not in the MDA-MB-231 or MDA-MB-435s cells as analyzed by RT-PCR/Southern blot hybridization (lower panel).

Prostasin mRNA expression is seen absent in two invasive human breast cancer cell lines while two non-invasive breast cancer cell lines express prostasin mRNA or protein. Analysis of prostasin expression in human breast cancer cell lines showed that the non-invasive MCF-7 and MDA-MB-453 cells express the prostasin mRNA while the highly invasive MDA-MB-231 and MDA-MB-435s cells do not express the prostasin mRNA (see FIG. 5). Expression of prostasin mRNA in normal human breast can be demonstrated by the presence of two GenBank™ normal human breast EST sequences coding for prostasin (Accession numbers R48653, and R48557). The MCF-7 cells also express the prostasin protein as determined by western blot analysis (again see FIG. 5). Prostasin down-regulation in prostate or breast cancer can be caused by promoter methylation or gene-specific mutation. Prostasin expression decreases with increasing prostate cancer grade and is absent in invasive prostate and breast cancer cell lines. The chromosomal locus where the human prostasin gene is, 11p11.2, however, is not known to be an LOH hot-spot in prostate cancer or in breast cancer. Epigenetic events (such as DNA methylation) may be an alternative mechanism of loss of expression for tumor suppressors or invasion suppressors. Refer now to FIG. 6 which shows promoter hypermethylation of the human prostasin gene in human prostate and breast cancer cell lines. Genomic DNA (5 μg) from the various cell lines (as indicated in the figure) were digested with the following restriction enzyme combinations, Xho I/BamH I (X/B, flanking cuts of the methylation-sensitive site), Xho I/BamH I/Msp I (X/B/M), or Xho I/BamH I/Hpa II (X/B/H). The digests were resolved in a 0.8% agarose gel and transferred to an Immobilon-N membrane for hybridization with a nick-translated prostasin promoter probe (bases 703-1,649 of the prostasin gene sequence U33446). The probe detects a promoter fragment of 1,275 bp, which is cut by the methylation-insensitive enzyme Msp I to yield a 951-bp fragment for all DNA samples. The methylation-sensitive isoschizomer Hpa II yields the 951-bp fragment in the CC-2555 (normal prostate epithelial cells), the LNCaP, and the MCF-7 samples, indicating the hypomethylated or unmethylated state of the prostasin promoter in these cells. For DU-145, PC-3, and MDA-MB453 DNA, both the 951-bp and the 1,275-bp fragments are generated in the methylation-sensitive digestion, suggesting incomplete methylation (one of two or more chromosomes) or clonal methylation in a sub-population of cells. For the MDA-MB-231 and MDA-MB-435, however, the Hpa II digestion did not yield the 951-bp but rather gave the 1,275-bp fragment. This homogeneous methylation pattern indicates that the Msp I/Hpa II site, at location-96 (relative to the transcription initiation site) of the prostasin promoter, is methylated (hypermethylated) in these DNA samples.

Signal intensity variation may be attributed to aneuploidy. In addition to the Hpa II site at base number 1326/-96 shown in FIG. 6, example 1 and 2 are provided, more extensively detailing the other prostasin gene promoter methylated sites and experimental methods for determining them. Such sites have been located at bp 615-807, 1156-266, 1,326-96 and 1,445/424. Nomenclature used in describing the nucleic acid sequences of the prostasin gene are described by Yu, et al in Structure and Chromosomal localization of the human prostasin (PRSS8) gene. Genomics 1996:32 pp. 334–40; page 336 of the Yu, et al reference is incorporated as FIG. 10 in the description of the present invention.

An examination of the prostasin gene promoter region for DNA methylation differences among human prostate and breast cancer cell lines has been made (see FIG. 6). We found that cells that express prostasin, normal prostate epithelial, LNCaP, and MCF-7, are unmethylated in the prostasin promoter while MDA-MB-435 showed heterogeneous prostasin promoter methylation. For cells that do not express prostasin, DU-145 and PC-3 showed heterogeneous prostasin promoter methylation while MDA-MB-231 and MDA-MB-435 showed homogeneous hypermethylation in the promoter region of the prostasin gene.

Two human prostate cancer cell lines that do not express prostasin, the highly invasive DU-145 and PC-3, show heterogeneous methylation in the promoter region of the prostasin gene. The result suggests that at least one of the two (or more) chromosome 16’s of these cell lines is methylated at the prostasin gene locus. The prostasin gene on the unmethylated chromosome may contain mutations that silenced the expression. An alternative explanation for the heterogeneous methylation pattern is that the methylation occurs in clonal cell populations, however, the lack of detectable prostasin mRNA in our RT-PCR-Southern blot analysis in the DU-145 and PC-3 cells argues against this possibility.

The significance of the finding on prostasin gene promoter hypermethylation in prostate or breast cancer is that the measurement of prostasin down-regulation as a cancer marker may be achieved by using a binary assay (yes-or-no), instead of a gradually decreasing quantity in the immunohistochemistry assay (which is quite arbitrary).

Re-expression of human prostasin protein in invasive human prostate and breast cancer cells reduces invasiveness in vitro. At this point, reference should be made to FIG. 7 which shows the prostasin protein expression and in vitro invasive properties of the DU-145 and the PC-3 transfecents. DU-145 or PC-3 cells transfected with either a vector
DNA (labeled as “vector”) or a prostasin cDNA construct (labeled as “prostasin”) were analyzed by a western blot analysis using a prostasin-specific antibody (upper panel) or subjected to an in vitro Matrigel chemoinvasion assay (lower panel) as described in (Liu DF, Rabbani SA. Induction of urinary plasminogen activator by retinoic acid results in increased invasiveness of human prostate cancer cells. PC-3. Prostate. 27(5):269–76, 1995). The expressed human prostasin protein (a 40-kDa band) was detected in the prostasin cDNA-transfected DU-145 or PC-3 cells, but not in the vector-transfected cells. In the Matrigel chemoinvasion assay, the vector-transfected cells are expressed as being 100% invasive (solid bar), the open bar represents the relative invasiveness of the human prostasin cDNA-transfected cells. The data were analyzed by a Student t-test using the StatView software (Abacus Concepts, Inc., Berkeley, Calif.).

It can be seen that Polyclonal DU-145 and PC-3 cells transfected with the human prostasin cDNA (designated DU-145/Pro, and PC-3/Pro, respectively) were confirmed to express the human prostasin protein, as shown in the western blot analysis of the cell lysate (FIG. 7, upper panel). The vector-transfected cells, designated DU-145/Vector or PC-3/Vector, respectively, were used as negative control in the western blot. A further examination of the DU-145/Pro and the PC-3/Pro cells by immunocytochemistry confirmed that 100% of the cells expressed the prostasin protein (data not shown). In in vitro Matrigel chemoinvasion assays (FIG. 7, lower panel), the invasiveness of DU-145/Pro cells was determined to be at 32% of that of DU-145/Nector cells (or, in the reduction of invasiveness was at 68%). The invasiveness of PC-3/Pro cells was determined to be at 58% of that of PC-3/Nector cells (or, the reduction of invasiveness was at 42%). We performed in vitro cell proliferation assays on DU-145/Pro vs. DU-145/Vector cells, and on PC-3/Pro vs. PC-3/Vector cells, but did not observe any difference between the growth rates of the prostasin cDNA-transfected or the vector-transfected cells over an 8-day period (data not shown).

Forced re-expression of human prostasin in two invasive human breast cancer cell lines reduced invasiveness. A full-length human prostasin cDNA under the control of an RSV promoter was transfected into the invasive breast cancer MDA-MB-231 and MDA-MB-435 cells. Reference should be made to FIG. 8 which shows prostasin protein expression and in vitro invasive properties of the MDA-MB-231 and the MDA-MB-435s transfectants. MDA-MB-231 and MDA-MB-435s cells transfected with either a vector DNA (labeled as “vector”) or a prostasin cDNA construct (labeled as “prostasin”) were analyzed by a western blot analysis using a prostasin-specific antibody (upper panel) or subjected to an in vitro Matrigel chemoinvasion assay (lower panel) as referenced in (Liu DF, Rabbani SA. Induction of urinary plasminogen activator by retinoic acid results in increased invasiveness of human prostate cancer cells. PC-3. Prostate. 27(5):269–76, 1995). The expressed human prostasin protein (a 40-kDa band) was detected in the prostasin cDNA-transfected MDA-MB-231 and MDA-MB-435s cells, but not in the vector-transfected cells. In the Matrigel chemoinvasion assay, the vector-transfected cells are expressed as being 100% invasive (solid bar), the open bar represents the relative invasiveness of the human prostasin cDNA-transfected cells. The data were analyzed by a Student t-test using the StatView software (Abacus Concepts, Inc., Berkeley, Calif.).

Stable, polyclonal, episomal transfectants were obtained and the expression of human prostasin protein was confirmed by western blot analysis (FIG. 8, upper panel). In in vitro Matrigel chemoinvasion assays, the invasiveness of either cell lines expressing human prostasin was reduced by 50% as compared to the vector-transfected controls (FIG. 8, lower panel).

Taken together, the foregoing evidences linking prostasin level reduction or prostasin absence to the invasiveness of prostate and breast cancer cell lines, or linking prostasin expression to reduced invasiveness. The evidence qualifies prostasin as an invasion suppressor, which thus is a marker for diagnosis of invasiveness of prostate and breast cancers, or as a therapeutic agent to treat invasive prostate and breast cancers.

Pathological grading by the Gleason system is performed after either surgery or biopsy, both highly invasive procedures, while blood tests such as that for the PSA prostate cancer marker can offer the hope of accurate diagnosis and prognosis without the harm of an invasive procedure. In practice, however, single markers suffer from an intrinsic limitation that the “positive” identifications are not always confirmed for the “diagnosed” disease. Biopsy is still required for a true positive identification of prostate cancer even in the case of the application of the PSA prostate cancer marker (Catalona W J, Partin A W, Slawin K M, Brawer M K, Flanagan R C, Patel A, Richic J P, deKemion J B, Walsh P C, Scardino P T, Lange P H, Subong E N, Parson R E, Gasior G H, Loveland K G, Southwick P C. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial [see comments]. JAMA. 279(19):1542–7, 1998). As stated above, it has been demonstrated in principle that many markers used in a multivariate approach may provide a highly accurate diagnosis (Greene G F, Kitadai Y, Pettaway Calif. von Eschenbach A C, Bucana C D, Fidler I J. Correlation of metastasis-related gene expression with metastatic potential in human prostate carcinoma cells implanted in nude mice using an in situ messenger RNA hybridization technique. American Journal of Pathology. 150(5):1571–82, 1997). From the standpoint of prostate cancer genetics, the multivariate approach is well supported by our current understanding. A serum protease structurally and genetically related to the PSA, the kH2 (human glandular kallikrein 2) has shown some promise of joining the list of markers applicable for prostate cancer diagnosis (Saedi M S, Hill T M, Kuus-Reichel K, Kumar A, Payne J, Mikolajczyk S D, Wolpert R L, Rittenhouse H G. The precursor form of the human kallikrein 2, a kallikrein homologous to prostate-specific antigen, is present in human sera and is increased in prostate cancer and benign prostatic hyperplasia. Clinical Chemistry. 44(10):2115–9, 1998). Structurally and in prostate gland biology, prostasin shares many common characteristics with both PSA and kH2, as being a secreted serine protease made in large abundance in prostate epithelial cells [Yu JX, Chao L, Chao J. Prostasin is a novel human serum proteinase from seminal fluid. Purification, tissue distribution, and localization in prostate gland. Journal of Biological Chemistry. 269(29):18843–8, 1994; Yu JX, Chao L, Chao J. Molecular cloning, tissue-specific expression, and cellular localization of human prostasin mRNA. Journal of Biological Chemistry. 270(22):13483–9, 1995]. While high-grade prostate cancer cells produce less PSA protein than normal prostate cells or low-grade prostate cancer cells (Hakaluhii L, Vihko P, Heintu P, Anto-Harmainen H, Soini Y, Vihko R. Evaluation of PAP and PSA gene expression in prostatic hyperplasia and prostatic carcinoma using northern-blot analyses, in situ hybridization and immunohistochemical stainings with
mono-clonal and tissue specific antibodies. International Journal of Cancer. 55(4):590–7, 1; Sakai H., Yogi Y., Minami Y., Yushita Y., Kanetake H., Saito Y. Prostate specific antigen and prostatic acid phosphatase immunoreactivity as prognostic; Sakai H., Yogi Y., Minami Y., Yushita Y., Kanetake H., Saito Y. Prostate specific antigen and prostatic acid phosphatase immunoreactivity as prognostic; the serum PSA levels in prostate cancer patients increased due to tissue damage caused by invasive cancer (Rittenhouse H. G. Finlay J. A., Mikolajczyk S. D., Partin A.W., Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. [Review] [457 refs] Critical Reviews in Clinical Laboratory Sciences. 35(4):275–368, 1998). By comparison, we also believe prostasin is in the circulation of prostate cancer patients. As a result, a blood test for the circulating prostasin to indicate the presence and/or the stage of prostate cancer would be highly useful. (as illustrated in FIG. 1). In principal, the feasibility of a blood test based on prostasin detection to indicate cancer has been demonstrated by Berteau et al. (1999). These authors (Berteau P., Laribi Eschewege P., Lebars I., Dumas F., Benoit G., Lorc S., Prostasin mRNA to detect prostate cells in blood of cancer patients. Clinical and Chemical Laboratory Medicine 37 (SS): SI 19, 1999), demonstrated a highly promising potential of using prostasin as a marker to detect circulating prostate epithelial cells, a sign of prostate tissue damage caused by invasive prostate cancer leading to the dissemination of prostate epithelial cells into the circulation. It is expected that a blood test for circulating prostasin to indicate the presence and/or the stage of breast cancer would be highly useful. (as illustrated in FIG. 2).

In summary of the invention, it has been taught herein that: protein prostasin as well as its mRNA and its gene promoter DNA methylation levels can be used to determine the invasiveness level of human carcinomas; and, provide a method of treating invasive human carcinomas by delivery thereto of a recombinant nucleic acid formed by a human prostasin nucleic acid incorporated into a selected gene delivery vector. Those teachings are repeated for emphasis in the following:

1. Immunohistochemistry studies of human prostate cancer specimens revealed a down-regulation of prostasin in high-grade tumors.

2. Using RT-PCR and western blot analyses, prostasin protein and mRNA expression were found in a non-invasive human breast cancer cell line, MCF-7, while invasive human breast cancer cell lines MDA-MB-231 and MDA-MB-435s were found not to express either the prostasin protein or the mRNA. A non-invasive human breast cancer cell line, MDA-MB-435s, was shown to express prostasin mRNA but not prostatin protein and:

3. Examination of the prostasin gene promoter in the human prostate and breast cancer cell lines by Southern blot analysis revealed heterogeneous methylation of the promoter in DU-145, PC-3 and MDA-MB453 cells, and homogeneous methylation of the promoter in MDA-MB-231 and MDA-MB-435s cells. The prostasin gene promoter in normal human prostate epithelial cells, the LNCAP and the MCF-7 cells was shown to be unmethylated. Transfection of DU-145 and PC-3 cells with a full-length human prostasin cDNA restored prostasin expression and reduced the in vitro invasiveness by 68% and 42%, respectively. Transfection of MDA-MB-231 and MDA-MB-435s cells with a full-length human prostasin cDNA restored prostasin expression and reduced the in vitro invasiveness by 50% for either cell line.

The preferred methods of separating sampled human carcinoma tissue from neighboring normal tissues is by laser capture micro-dissection.

The following examples are provided for the purpose of illustration and not limitation:

Example 1

Prostasin Promoter DNA Methylation

Cell Culture Maintenance

All cell culture media, sera and supplements were purchased from Life Technologies (Gaithersburg, Md.) except for those otherwise noted.

A normal human mammary epithelial cell primary culture (catalog number CC-255) was obtained from Clonetics (San Diego, Calif.) and maintained in the mammary epithelial basal medium (supplemented with bovine pituitary extract, recombinant human epidermal growth factor, bovine insulin and hydrocortisone) according to the suppliers protocols. The Culture was kept at 37°C. with 5% CO₂ and used for experiments at the 9th overall passage.

Human breast carcinoma cell lines MCF-7, MDA-MB-453, MDA-MB-231 and MDA-MB-435 were obtained from the American Type Culture Collection (ATCC, Manassas, Va.). The MCF-7 cells were maintained in DMEM supplemented with 10% FBS and kept at 37°C for experiments at the 9th overall passage.

Human breast carcinoma cell lines MCF-7, MDA-MB-453, MDA-MB-231 and MDA-MB-435 were obtained from the American Type Culture Collection (ATCC, Manassas, Va.). The MCF-7 cells were maintained in DMEM supplemented with 10% FBS and kept at 37°C with 5% CO₂. The MDA-MB-231 and MDA-MB-453 cells were maintained in Lebovitz-15 (L-15) medium supplemented with 10% FBS and kept at 37°C without CO₂. The MDA-MB-435 cells were maintained in L-15 medium supplemented with 15% FBS and 10 μg/ml bovine insulin and kept at 37°C without CO₂.

Transfection Of Cell Lines With Plasmid DNA And Selection Of Stable Transfectants

Construction of a plasmid containing a full-length human prostasin cDNA under the control of a Rous sarcoma virus (RSV) promoter and transfection of cells were carried out as described in Chen, L.M., Hodge, G.B., Guarda, L.A. et al. Down-Regulation of Prostasin Serine Protease, A potential Invasion Suppressor in Prostate Cancer, Prostate 2001, 48:93–103. Briefly, 1,000,000 MDA-MB-231 or MDA-MB-435s cells were resuspended in 0.3 ml of the culture medium and mixed with 50 μg of plasmid DNA dissolved in 0.1 ml of sterile distilled water and pulsed at 200 volts, 1,600 μA, 72 ohms and 500 V capacitance setting on a BTX-600 Electro-cell manipulator (Genetronics, San Diego, Calif.). Selection of transfectants was carried out in the presence of 800 μg/ml G418 (final concentration) until colonies appeared. (5–7 days) Colonies (~200 in number) were then dispersed via trypsinization and maintained in G418 containing culture medium without colony-isolation. Cells transfected with the human prostasin cDNA construct were assayed in Western blot analysis for expression of the prostasin protein, using vector-transfected cells as controls.

RNA Preparation And Analysis By RT-PCR/Southern Blot

Cells grown to 80% confluence (in 60 mm tissue culture dish) were lysed directly with 1 ml of the Trizol Reagent (life Technologies) and RNA was isolated according to the manufacturer’s protocol. The human prostasin-specific RT-PCR/Southern blot analysis was performed as described in Yu, J. X., and Chao, J. Molecular Cloning, tissue-specific expression and cellular localization of human prostasin.
mRNA. J Biol Chem 1995; 270:12483-4. One microgram of total RNA was used in the RT-PCR with 2 human prostasin gene-specific oligonucleotide primers, and a Southern blot of the resolved RT-PCR samples was probed with a third prostasin gene-specific oligonucleotide, detecting an amplified 232 bp fragment. A co-amplification of the β-actin mRNA for control of RNA quality and quantity was performed as described in Chen Hodge and Guarda, et al.

Western Blot Analysis

Cells grown to 80–100% confluence were washed 3 times in 1xPBS (pH 7.4) and then lysed in RIPA buffer (1xPBS, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). The total lysate were centrifuged at 14,000 rpm for 30 minutes at 4°C to remove the pellet. Protein concentration was determined using a DC (detergent-compatible) protein assay kit (Bio-Rad, Hercules, Calif.). The samples were then subjected to SDS-PAGE followed by Western blot analysis using a prostasin-specific antibody. Briefly, cell lysate were resolved in 10% gels under reducing conditions before electrotransfer to nitrocellulose (NC) membranes (Fisher Scientific, Pittsburgh, Pa.). Upon complete protein transfer, the NC membranes were blocked in BLOTTO (5% nonfat milk made with Tris-buffered saline; 0.1% Tween-20, pH 7.6), incubated with the prostasin-specific antibody diluted at a ratio of 1:2000 in BLOTTO, followed by an incubation with the secondary antibody, goat antirabbit IgG conjugated to HRP (horseradish peroxidase; used at 1:10,000) (Sigma-Aldrich, St. Louis Mo.). The bound secondary antibody was detected using enhanced chemi-luminescence (ECL) reagents (Perico, Rockford, Ill.) according to the suppliers recommendations. All procedures were performed at room temperature, and the membranes were washed between steps.

Matrigel Chemo Invasion Assay

The Matrigel chemo invasion assay was carried out essentially as described in Chen, L. M., Hodge, G B, Guarda, LA et al. Down-Regulation of prostasin serine protease, a potential invasion suppressor in prostate cancer. Prostate 2001:48:93-103, incorporated herein. Basement membrane Matrigel stock (10 mg/ml; Collaborative Biochemical, Bedford, Mass.) was thawed overnight on ice in a refrigerator (0–4°C). Transwell invasion chambers (Coster, Cambridge, Mass.) with 8 μm pore polycarbonate filters (growth area 0.33 cm²) were coated with Matrigel (50 μg/filter, a 1:3 dilution of the stock with chilled serum-free medium was used for coating). The gels were solidified by incubating the coated filters at 37°C for 60 min in a moist chamber before use. The lower chambers of the Transwell plates were filled with 0.6 ml serum-free medium (1-15, supplemented with 25 μg/ml fibronectin (Sigma-Aldrich)). Fifty thousand cells in 0.1 ml serum-free OPTI-MEM 1 medium were placed onto each Matrigel-coated filter. The filter cartridge was then inserted into the lower chamber and the assays were performed at 37°C for 24 hours (MDA-MB-435s transfectants) or 72 hour (MDA-MB-231 transfectants). After removing the medium, the filters were washed 3 times in 1xPBS, fixed at room temperature for 20 min in 4% paraformaldehyde made with 0.1 M sodium phosphate buffer (pH 7.4) and then washed 3 times with the sodium phosphate buffer. The filters were then stained with 1% toluidine blue (LabChem, Pittsburgh, Pa.) for 2 min. The cells on the Matrigel surface were removed with a Q-tip and all cells on the underside of the filters were counted under a light microscope after mounting the filter on a glass slide. All invasion assays were done in triplicate for at least 3 times.

Genomic Southern Blot Analysis Of Prostasin Promoter Methylation

High molecular weight genomic DNA was isolated from various cell lines as described in Chai, K X, Ward, D C, Chao, J, et al Molecular Clonings Sequence Analysis and Chromosomal Localization of the Human Protease Inhibitor 4 (kallistatin) gene (P14) Genomics 1994; 23:370-8. Genomic DNA from each cell line was digested with the following restriction enzyme combinations: Xho I/BamH I (X/B), Xho I/BamH I/Hind III/BamH I (H/B/I), Xho I/BamH I/Asc I (X/B/A), Xho I/BamH I/L.Sba I (X/B/S),Xho I/Bam H I/Msp I/(X/B/M) or Xho I/Bam H I/Hpa II/(X/B/H) using 10 μg of DNA per digestion combination. The X/B cutting sites flank the CpG sites be to be investigated for differential methylation by the methylation-sensitive restriction endonucleases. The digests were resolved in a 0.8% agarose gel and analyzed by Southern blot hybridization as described in Chai, K X, Ward, D C, Chao, J et al. A nick translated prostasin promoter probe (bases 703-1,649) of the prostasin gene sequence, Genbank accession number U33446) was used for the hybridization. The base numbering of the prostasin gene sequence was described in Yu et al.

Demethylation of Prostasin Gene Promoter and Reactivation of Prostasin Expression

For demethylation of prostasin gene promoter, MDA-MB-231 and MDA-MB-435s cells were seeded in 100 mm dishes at an initial density of 50% confluence and cultured in the presence of 500 nM 5-aza-2'-deoxycytidine (5-aza-2'dC; Sigma-Aldrich) for 8 days with renewal of medium and 5-aza-2'dC performed at 2 day intervals. Genomic DNA was then isolated for Southern blot analysis as described. For reactivation of prostasin expression, MDA-MB-231 and MDA-MB-435s cells were seeded in 60 mm dishes at 80% confluence and cultured in the presence of 500 nM 5-aza-2'dC for 24 hr, and were then treated for an additional 24 hr with either 1 μM trichostatin A (TSA; Sigma Aldrich) or an equal volume of 95% ethanol used to dissolve TSA. For measuring time-dependence of prostasin gene reactivation, cells were treated as described above but were harvested at 6 or 12 hr after the addition of TSA. RNA was isolated for prostasin-specific RT-PCR/Southern blot analysis as described. The MCF-7 and the MDA-MB-453 cell lines were subjected to the same 5-aza-2'dC/TSA treatment and prostatin RT-PCR Southern blot analysis procedures.

Results

Prostasin Expression In Breast Cancer Lines

Expression of prostasin protein and mRNA in Normal Human Mammary Epithelial Cells (NHMEC) and human breast carcinoma cell lines was examined by Western blot analysis using a prostasin-specific antibody or RT-PCR/Southern blot analysis as described. For this experiment 4 breast carcinoma cell lines based on their differences in invasiveness and metastatic potential were chosen. The MCF-7 cell line is poorly invasive to noninvasive, tumorigenic but nonmetastatic. The MDA-MB-435 cells are poorly tumorigenic and nonmetastatic. The MDA-MB-231 and MDA-MB-435 cells are both highly invasive and highly metastatic.

As was shown in FIG. 5 (middle panel) a 232 bp amplified prostasin message was detected in total RNA of NHMEC, MCF-7 and MDA-MB-453 but not MDA-MB-231 or MDA-MB-435s. A co-amplification of β-actin message is shown in the bottom panel of FIG. 5 to demonstrate the quality and quality of total RNA used in each RT-PCR. In addition to the expected 232 bp prostasin amplification product, a faint band of retarded mobility was also detected in the Southern blot analysis. This retarded band does not represent a new
prostasin message, but rather is a single-stranded form of the expected prostasin PCR product. In a separate experiment, we were able to remove this single-stranded form by treating the PCR products with SI nuclease prior to electrophoresis (data not shown).

Re-expression of recombinant human prostasin in invasive breast cancer cells reduced in vitro invasiveness

Following the electroporation and drug selection ~200 colonies formed for each transfected cell type. All colonies for each transfected cell type were kept in a mixed culture as polyclonal transfectants for the ensuing experiments. Polyclonal MDA-MB-231 and MDA-MB-435s cells transfected with the human ~320 bp cDNA were confirmed to express the recombinant human prostasin protein, as shown by Western blot analysis of the cell lysate (FIG. 8, top panel). The vector transfected cells were used as negative control in the Western blot analysis.

In the in vitro Matrigel chemoinvasion assays shown in FIG. 8, bottom panel) transfected MDA-MB-231 or MDA-MB-435s cells expressing the recombinant human prostasin protein showed a significantly reduced level of invasiveness, at 50% of that of their vector transfected controls, respectively.

Prostasin Gene Promotor Is Hypermethylated In Invasive Breast Cancer Lines

Examination of the prostasin gene locus by genomic Southern blot-RFLP (restriction fragment length polymorphism) analysis did not reveal any evidence of gene loss or gross rearrangement in the cell lines that did not show prostasin expression, namely, MDA-MB435 (data not shown). Consideration of the possibility of prostasin gene-silencing by epigenetic mechanisms was also considered. Polyadenylation of cytome residues in the 5’-CpG islands is often involved in long-term silencing of certain genes during mammalian development and in the progression of cancers. An examination of the prostasin promoter and exon I region sequence (GenBank accession number U33446) identified 28 CpG dinucleotides in a segment defined by an Xho I site (base number 374 of U33446 or -1,048 relative to the transcription initiation site and a BamHI site (base number 1,649 of U33446 or +228 relative to the transcription initiation site) (FIG. 9a). A GC enriched domain containing 2 consensus G/C boxes with the sequence GGGCGG was identified as encompassing the transcription initiation site. This GC enriched domain extends from base number 1,101 to the BamHI site (1,649) as a length of 549 bp and has a GC content of 58.6%. The 549 bp GC-enriched domain contains 19 CpG dinucleotides, but failed to qualify as a true CpG island by the standards of Gardiner-Garden, M., and Frommer, M. CpG islands is homogenously methylated.

In the NHMEC digestion with Xho I and BamHI digestion yielded a 1,275 bp prostasin promoter band seen in the Xho I/BamH I digestion (FIG. 9b) indicating that the -807 Hpa I site CpG is homogenously methylated.

In NHMEC and MCF-7 cells the 1,275 bp Xho I/BamHI I band can be digested by the methylation-insensitive Msp I as well as by the methylation sensitive Hpa II yielding an 951 bp band, indicating that the CpG at the -96 M/H restriction site is homogenously unmethylated. In MDA-MB-435, the -1,275 bp band can be fully digested by Msp I, but only partially by Hpa II indicating heterogeneous methylation of the -96 M/H site CpG. In MDA-MB-231 and MDA-MB-435s, the -1,275 bp band was undigested by Hpa II while having been completely digested by Msp I, indicating that the -96 M/H site CpG is homogenously methylated.

Overall, in the prostasin-expressing cells the NHMEC is the least methylated in this region and the methylation is restricted to beyond ~266 relative to the transcription initiation site. The prostasin gene promoter-exon I region in MCF-7 and MDA-MB-453 cells is methylated to a higher extent than that in the NHMEC. In the MCF-7, methylation is restricted to beyond ~966, while in the MDA-MB-453, methylation is observed at the ~96 position and beyond. In the cells that do not express prostasin, the promoter-exon I region is the most heavily methylated. While in MDA-MB-231 the methylation is still restricted to the 5’ flanking region, all CpG sites examined, including 1 of exon I (+24 Aci I site CpG) were found to be homogenously methylated in the MDA-MB-435s cells. The results showed that the prostasin gene promoter-exon I region CpG methylation patterns correlate with the absence of prostasin expression in the human breast cells examined.

Prostasin Expression Was Reactivated By Demethylation And Histone Dacetylase Inhibition In The Invasive And Metastatic Breast Cancer Cell Lines

Methylated promoter DNA may contribute to repression of gene expression by interfering with the binding of transcription factors, or by interacting with various methyl-CpG binding proteins. Such as the MeCP1 complex, McCP2, and the MBD’s depending on cell type, sequence of the methylated regulatory region and binding of other transcription factors. Histone deacetylases are recruited by the methyl-CpG binding proteins to co-repress gene expression via chromatin restructuring. Histone deacetylases act synergistically with DNA methylation in the co-repression, with DNA methylation being the dominant force for stable maintenance of gene silencing in cancer. Treatment of cancer cells with the demethylating agent 5-aza-2'-deoxycytidine (5-aza-2’dC) may restore a minimal expression for genes silenced by promoter methylation and the combined treatment of 5-aza-2’-dC with the histone deacetylase inhibitor, trichostatin A (TSA) may further stimulate the restored expression. Investigation of demethylation and/or histone deacetylase inhibition could reactiva prostasin gene expression in the MDA-MB-231 and MDA-MB-435s cells proceeded as follows:

After 8 days of treatment with the DNA methyltransferase inhibitor 5-aza-2’-dC, a significant level of demethylation was observed in the prostasin promoter-exon 1 region in the MDA-MB-435s cells, as genomic DNA of this region in these cells was partially digested by Hha I at ~807, Aci I at ~320, BsaAl at ~266 and Hpa I at ~96 (**+FIG. 9c). The +24 Aci I site was also partially digested in MDA-MB-435s following demethylation (FIG. 9c) Examination of total RNA of these cells by RT-PCR/Southern blot analysis following the 8-day 5-aza-2’-dC treatment, however, failed to detect prostasin mRNA expression (data not shown).

Investigation of the combined effect of treating the cells with 5-aza-2’-dC and TSA, an inhibitor of histone deacetylase followed. In RT-PCR/Southern blot analysis, a time-
dependant reactivated expression of prostasin mRNA was observed in both the MDA-MB-231 and the MDA-MB-453 cells after a treatment with 5-aza-2'-dC for 24 hr followed by 95% ethanol (solvent for TSA) did not result in prostasin mRNA re-expression. A co-amplification of the β-actin message confirmed the quality and quantity of the total RNA used in each RT-PCR. When MCF-7 and MDA-MB-453 cells were subjected to the same 5-aza-2'-dC/TSA treatment and prostasin RT-PCR/Southern blot analysis procedures, no increase of prostasin mRNA expression was observed for either cell line.

FIG. 9a The solid horizontal line represents the promoter region of the human prostasin gene (Genbank U33464) The Xho I site is located at Base 1,649 of the U33466 sequence respectively. The methylation sensitive restriction sites used for differential methylation analysis, Hha (615/-807), AcI I (1,102/-320 and 1,445/424), BsaA I (1,156/-266) andMsp l/Hpa II (1,326/-96) are indicated by arrows. Four additional AcI sites and a second dMsp Hpa Ii site are present between the +24 AcI site and the Bam H I sites but not shown in the figure since they do not affect the results of the genomic DNA Southern blot analysis. The transcription initiation site is indicated by the triangle on an extended vertical bar. The vertical bars map the location of the consensus G/C boxes. The extended vertical bar with open square boxes indicate the locations of the consensus G/C boxes. The location of the probe used in the genomic DNA Southern blot analysis is shown by the open rectangular box.

FIG. 9b Panels of genomic DNA Southern blot analysis results are as indicated for each cell type. Restriction endonucleases used in each digestion mixture are indenitified as follows: X, Xho I, B, Bam H I, Hh, Hha I, A, AcI, B, BsaA I, M, Msp I, H, Hpa II. Hha I, AcI I, BsaA I and Hpa II only cut unmethylated DNA while Msp I, an isoschizomor of Hpa II, cuts unmethylated or methylated DNA. Genomic DNA (10 ug) from each cell type was cut with X/B or with X/B/M to serve as controls for the detection of differential methylation. In the upper panel, prostasin promoter DNA will yield a 1,275 bp X/B digestion fragment. When Hha I was added to the X/B digestion mixture a fragment of 1,037 bp was expected if the ~807 Hha site Cpg was not methylated. When AcI I was added to the X/B digestion mixture, 3 smaller bands might be expected depending on CpG methylation states at ~320 and +24 AcI sites. They are 1,072 bp(from Xho I to +24 AcI I) 727 bp (from Xho I to ~320 AcI I) and 345 bp (from ~320 AcI to +24 AcI I) in length, respectively. When BsaA I was added to the X/B digestion mixture, 2 smaller bands might be expected depending on CpG methylation state at the ~266 Bsa A I. They are 782 bp (from Xho I to BsaA I) and 493 bp (from BsaA I to Bam H I) in length, respectively (indicated by asterisks in the figure). In the lower panel, the upper arrow points to the X/B fragment, while the lower arrow points to the fragment that is generated by Msp I, regardless of DNA methylation, or by Hpa II, only when DNA is unmethylated.

FIG. 9c Cells treated with 5-aza-2' dC for 8 days were harvested for genomic DNA isolation and Southern blot analysis as described. The arrowhead indicates the 1,037 bp Hha I-BamH I fragment when the ~807 ha I site was cut by enzyme following DNA demethylation.
tive to the transcription initiation site. The prostasin gene promoter-exon 1 region in LNCaP cells is methylated to a higher extent than that in the PrEC. In LNCaP, which also express both the prostasin mRNA and protein, methylation is only observed at beyond −96. In DU-145, which does not express either the prostasin protein or mRNA, the promoter-exon 1 region is the most heavily methylated. While in DU-145, the methylation is limited to the 5 flanking region, all CpG sites examined, including one of exon 1 (+24 Aci I site-CpG) show heterogeneous to homogeneous methylation in the PC-3 cells. The prostasin gene promoter-exon region CpG methylation patterns correlate with the absence of prostasin expression in the human prostate cells.

Table I details the methylation state of the prostate cells. As shown with the breast cancer cells the methylation state of the prostasin promoter region may be examined for a potential diagnostic application to indicate prostate cancer invasiveness. For example, the prostasin gene promoter methylation state at the −96M/H CpG site, for example, is seen methylated in invasive cell types.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Pro-</th>
<th>Hha 1 (-807)</th>
<th>Aci 1 (-320)</th>
<th>Bsa A 1 (-266)</th>
<th>MspI/</th>
<th>Hpa 1 I (-96)</th>
<th>Aci 1 (+24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrEC</td>
<td>Yes</td>
<td>Methyl</td>
<td>Hetero-Methyl</td>
<td>Un-Methyl</td>
<td>Un-</td>
<td>Un-Methyl</td>
<td>Hetero-Methyl</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Yes</td>
<td>Methyl</td>
<td>Methyl</td>
<td>Un-Methyl</td>
<td>Un-</td>
<td>Un-Methyl</td>
<td>Methyl</td>
</tr>
<tr>
<td>DU-145 No</td>
<td>No</td>
<td>Methyl</td>
<td>Methyl</td>
<td>Hetero-Methyl</td>
<td>Hetero-Methyl</td>
<td>Methyl</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>No</td>
<td>Methyl</td>
<td>Methyl</td>
<td>Hetero-Methyl</td>
<td>Methyl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table Legend:
Methyl = homogeneously methylated
Hetero-Methyl = heterogeneously methylated
Un-Methyl = homogeneously unmethylated

In investigating whether methylation in the promoter is causal to the lack of prostasin gene expression in the DU-145 and PC-3 cells, as in the breast cancer cells, treatment of these cell lines for 8 days with the DNA methyltransferase inhibitor 5-aza-2'-dC resulted in significant level of demethylation but no prostasin mRNA expression was detected in an RT-PCR/Southern blot analysis of the DNA demethylated cells. Treatment of the cells with the combination of 5-aza-2'-dC and trichostatin A (TSA) an inhibitor or histone deacetylase however restored prostasin mRNA expression in DU-145 and PC-3 cells (data not shown) When investigating whether the prostasin protein was expressed in the 5-aza-2'dC/TSA treated cells by western blot analysis, the result was negative. It appears that although promoter DNA methylation is causal to absence of prostasin expression in the invasive prostate cancer cells, the reactivated expression represents only the basal expression, similar to what was observed in the breast cancer cells.

Examples I and II demonstrate that the methylation patterns of the same specific sites in both breast cancer and prostate cancer provide markers for invasiveness.

While the invention has been described, disclosed, illustrated and shown in various terms of certain embodiments or modifications which it has presumed in practice, the scope of the invention is not intended to be, nor should it be deemed to be, limited thereby and such other modifications or embodiments as may be suggested by the teachings herein are particularly reserved especially as they fall within the breadth and scope of the claims here appended.

We claim:
1. A method for determining whether human prostate or breast carcinomas are non invasive, comprising sampling human breast or prostate carcinomas and determining whether the prostasin gene promoter DNA is methylated at bp 1,156/-266, wherein the absence of methylation indicates a non-invasive carcinoma.
2. The method, as in claim 1 wherein the sampling of the carcinoma tissue includes the step of separating the human carcinoma tissue from neighboring normal tissue using a laser capture micro-dissection.
3. The method, as in claim 1 wherein the step of determining gene promoter DNA methylation patterns includes applying prostasin-promoter-specific oligonucleotide primers in a PCR to determine the prostasin gene promoter DNA methylation patterns in the sampled tissue.
4. A method for determining which cancer patients do not require chemotherapeutic treatment, comprising taking a biopsy of a prostate or breast tumor, and determining the non-invasiveness of the carcinoma tissue based on the absence of methylation of the prostasin gene promoter DNA at bp 1,156/-266.
5. A method for determining whether human prostate carcinomas are non invasive, comprising sampling human prostate carcinomas and determining whether the prostasin gene promoter DNA is methylated at bp 1,156/-266, wherein the absence of methylation indicates a non-invasive carcinoma and the presence of methylation indicates invasive carcinoma.
6. The method of claim 5 wherein the sampling of the carcinoma tissue includes the step of separating the human carcinoma tissue from neighboring normal tissue using a laser capture micro-dissection.
7. The method of claim 5 wherein the step of determining gene promoter DNA methylation patterns includes applying prostasin-promoter-specific oligonucleotide primers in a PCR to determine the prostasin gene promoter DNA methylation patterns in the sampled tissue.

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