(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 August 2008 (28.08.2008)

PCT

(10) International Publication Number
WO 2008/102906 A1

(51) International Patent Classification:
C12N 15/09 (2006.01) *G01N 33/50* (2006.01)
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/JP2008/053133

(22) International Filing Date:
19 February 2008 (19.02.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/902,533 20 February 2007 (20.02.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) **Title:** HSPC-HRPC TRANSITION GENES

(57) **Abstract:** Objective methods for diagnosing hormone-refractory prostate cancer (HRPC) are described herein. In one embodiment, the diagnostic method involves the determining a expression level of HRPC-associated gene that discriminate between HRPC and HSPC. The present invention further provides methods of screening for therapeutic agents useful in the treatment of HRPC, methods of treating HRPC.

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DESCRIPTION

HSPC-HRPC TRANSITION GENES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/902,533, filed on February 20, 2007, the entire contents of which is hereby incorporated herein by reference for all purposes.

TECHNICAL FIELD

The present invention relates to methods of detecting and diagnosing hormone-refractory prostate cancer (HRPC) as well as methods of treating and preventing hormone-refractory prostate cancer.

BACKGROUND ART

Prostate cancer (PC) is the most common malignancy in males and the second-leading cause of cancer-related death in the United States and Europe (Gronberg H., Lancet 2003; 361:859-64.). The incidence of prostate cancer has been increasing significantly in most developed countries due to prevalence of a western-style diet and the explosion of the aging population (Gronberg H. Lancet 2003; 361:859-64., Hsing AW & Devesa SS. Epidemiol Rev. 2001;23(1):3-13.). Screening using serum prostate-specific antigen (PSA) has lead to a dramatic improvement in the early detection of prostate cancer, and has resulted in an increase of the proportion of patients with localized disease that could be cured by surgical and radiation therapies (Gronberg H. Lancet 2003; 361:859-64., Hsing AW & Devesa SS. Epidemiol Rev. 2001;23(1):3-13.). However, 20-30% of these PC patients still suffer from a relapse of the disease (Feldman BJ & Feldman D. Nat Rev Cancer. 2001 Oct;1(1):34-45., Han M, *et al.*, J Urol. 2001 Aug;166(2):416-9., Isaacs W, *et al.*, Cancer Cell 2002; 2:113-6.).

The androgen/androgen receptor (AR) signaling pathway plays a central role in development of PC, and PC growth is usually androgen-dependent at a relatively early stage (Feldman BJ & Feldman D. Nat Rev Cancer. 2001 Oct;1(1):34-45., Han M, *et al.*, J Urol. 2001 Aug;166(2):416-9., Isaacs W, *et al.*, Cancer Cell 2002; 2:113-6.). Hence, most of the

patients with relapsed or advanced disease respond well to androgen-ablation therapy, which suppresses testicular androgen production by surgical castration or by administration of one or more agonist(s) to luteinizing hormone-releasing hormone (LH-RH) and anti-androgen drugs. Nonetheless, the PC cells eventually acquire an androgen-independent and more aggressive phenotype that has been termed hormone-refractory prostate cancer (HRPC), for which no effective anti-cancer drug or therapy is available at present (Feldman BJ & Feldman D. Nat Rev Cancer. 2001 Oct;1(1):34-45., Han M, *et al.*, J Urol. 2001 Aug;166(2):416-9., Isaacs W, *et al.*, Cancer Cell 2002; 2:113-6.).

Several studies using *in vitro* PC cell lines and mouse models have shown that the progression to HRPC could be associated with increased levels of AR expression, implicating that AR down-regulation by means of small interfering RNA (siRNA) or other methods can suppress tumor growth in vivo even in HRPC cells (Gregory CW, *et al.*, Cancer Res. 1998 Dec 15;58(24):5718-24., Chen CD, *et al.*, Nat Med. 2004 Jan;10(1):33-9. Epub 2003 Dec 21., Zegarra-Moro OL, *et al.*, Cancer Res. 2002 Feb 15;62(4):1008-13.). The AR gene was over-expressed in most of HRPC cells, in 10-20% of which amplification of the AR gene was observed (Linja MJ, *et al.*, Cancer Res. 2001 May 1;61(9):3550-5.).

In addition, a subset (less than 10%) of HRPC cells was found to have somatic mutations in the AR gene, which could enhance the ligand response (Taplin ME, *et al.*, J Clin Oncol. 2003 Jul 15;21(14):2673-8.). As a consequence, expression of a number of AR-regulated genes was activated under androgen depletion (Feldman BJ & Feldman D. Nat Rev Cancer. 2001 Oct;1(1):34-45., Debes JD & Tindall DJ. N Engl J Med. 2004 Oct 7;351(15):1488-90., Holzbeierlein J, *et al.*, Am J Pathol. 2004 Jan;164(1):217-27., Balk SP. Urology. 2002 Sep;60(3 Suppl 1):132-8; discussion 138-9.).

Furthermore, the AR pathway in HRPC cells was considered to rely on alterations in growth factors such as insulin-like growth factor (Culig Z, *et al.*, Cancer Res. 1994 Oct 15;54(20):5474-8.), HER-2 (Craft N, *et al.* Nat Med. 1999 Mar;5(3):280-5.), and cytokines such as interleukin-6 (Hobisch A, *et al.*, Cancer Res. 1998 Oct 15;58(20):4640-5.), which can modify the AR activity. Over-expression of these growth factors and co-activators in HRPC cells might change cancer cells to be independent of the AR signaling (Debes JD & Tindall DJ. N Engl J Med. 2004 Oct 7;351(15):1488-90., Grossmann ME, *et al.* J Natl Cancer Inst. 2001 Nov 21;93(22):1687-97.). Despite the latest advances in molecular analysis, the mechanisms by which PC cells survive and acquire a more aggressive phenotype after

androgen-ablation therapy are still not well understood. Hence, no effective treatment to HRPC patients is available at present.

Studies designed to reveal mechanisms of carcinogenesis have already facilitated identification of molecular targets for anti-tumor agents. For example, farnesyltransferase inhibitors (FTIs) which were originally developed to inhibit the growth-signaling pathway related to Ras, whose activation depends on posttranslational farnesylation, has been effective in treating Ras-dependent tumors in animal models (Sun J, *et al.*, Oncogene. 1998 Mar;16(11):1467-73.). Clinical trials on humans using a combination of anti-cancer drugs and the anti-HER2 monoclonal antibody, trastuzumab, have been conducted to antagonize the proto-oncogene receptor HER2/neu; and have been achieving improved clinical response and overall survival of breast-cancer patients (Molina MA, *et al.*, Cancer Res. 2001 Jun 15;61(12):4744-9.). A tyrosine kinase inhibitor, STI-571, which selectively inactivates bcr-abl fusion proteins, has been developed to treat chronic myelogenous leukemias wherein constitutive activation of bcr-abl tyrosine kinase plays a crucial role in the transformation of leukocytes. Agents of these kinds are designed to suppress oncogenic activity of specific gene products (O'Dwyer ME & Druker BJ. Curr Opin Oncol. 2000 Nov;12(6):594-7.). Therefore, gene products commonly up-regulated in cancerous cells can serve as potential targets for developing novel anti-cancer agents.

It has been demonstrated that CD8+ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumor-associated antigens (TAAs) presented on MHC Class I molecule, and lyse tumor cells. Since the discovery of the MAGE family as the first example of TAAs, many other TAAs have been discovered using immunological approaches (Boon, Int J Cancer 54: 177-80 (1993); Boon and van der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen *et al.*, Science 254: 1643-7 (1991); Brichard *et al.*, J Exp Med 178: 489-95 (1993); Kawakami *et al.*, J Exp Med 180: 347-52 (1994)). Some of the discovered TAAs are now in clinical development as targets of immunotherapy. TAAs discovered so far include MAGE (van der Bruggen *et al.*, Science 254: 1643-7 (1991)), gp100 (Kawakami *et al.*, J Exp Med 180: 347-52 (1994)), SART (Shichijo *et al.*, J Exp Med 187: 277-88 (1998)), and NY-ESO-1 (Chen *et al.*, Proc Natl Acad Sci USA 94: 1914-8 (1997)). On the other hand, gene products which had been demonstrated to be specifically over-expressed in tumor cells, have been shown to be recognized as targets inducing cellular immune responses. Such gene products include p53 (Umano *et al.*, Brit J Cancer 84: 1052-7 (2001)), HER2/neu (Tanaka *et*

al., Br J Cancer 84: 94-9 (2001)), CEA (Nukaya *et al.*, Int J Cancer 80: 92-7 (1999)), and others.

In spite of significant progress in basic and clinical research concerning TAAs (Rosenberg *et al.*, Nature Med 4: 321-7 (1998); Mukherji *et al.*, Proc Natl Acad Sci USA 92: 8078-82 (1995); Hu *et al.*, Cancer Res 56: 2479-83 (1996)), only a limited number of candidate TAAs for the treatment of adenocarcinomas are available. TAAs abundantly expressed in cancer cells, and at the same time which expression is restricted to cancer cells would be promising candidates as immunotherapeutic targets. Further, identification of new TAAs inducing potent and specific antitumor immune responses is expected to encourage clinical use of peptide vaccination strategy in various types of cancer (Boon and van der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen *et al.*, Science 254: 1643-7 (1991); Brichard *et al.*, J Exp Med 178: 489-95 (1993); Kawakami *et al.*,J Exp Med 180: 347-52 (1994); Shichijo *et al.*, J Exp Med 187: 277-88 (1998); Chen *et al.*, Proc Natl Acad Sci USA 94: 1914-8 (1997); Harris, J Natl Cancer Inst 88: 1442-5 (1996); Butterfield *et al.*, Cancer Res 59: 3134-42 (1999); Vissers *et al.*, Cancer Res 59: 5554-9 (1999); van der Burg *et al.*, J Immunol 156: 3308-14 (1996); Tanaka *et al.*, Cancer Res 57: 4465-8 (1997); Fujie *et al.*, Int J Cancer 80: 169-72 (1999); Kikuchi *et al.*, Int J Cancer 81: 459-66 (1999); Oiso *et al.*, Int J Cancer 81: 387-94 (1999)).

It has been repeatedly reported that peptide-stimulated peripheral blood mononuclear cells (PBMCs) from certain healthy donors produce significant levels of IFN-γ in response to the peptide, but rarely exert cytotoxicity against tumor cells in an HLA-A24 or -A0201 restricted manner in ^{51}Cr-release assays (Kawano *et al.*, Cance Res 60: 3550-8 (2000); Nishizaka *et al.*, Cancer Res 60: 4830-7 (2000); Tamura *et al.*, Jpn J Cancer Res 92: 762-7 (2001)). However, both of HLA-A24 and HLA-A0201 are one of the more prevalent HLA alleles amongst Japanese, as well as Caucasian individuals (Date *et al.*, Tissue Antigens 47: 93-101 (1996); Kondo *et al.*, J Immunol 155: 4307-12 (1995); Kubo *et al.*, J Immunol 152: 3913-24 (1994); Imanishi *et al.*, Proceeding of the eleventh International Histocompatibility Workshop and Conference Oxford University Press, Oxford, 1065 (1992); Williams *et al.*, Tissue Antigen 49: 129-33 (1997)). Thus, antigenic peptides of carcinomas presented by these HLAs can be especially useful for the treatment of carcinomas among Japanese and Caucasian. Further, it is known that the induction of low-affinity CTL *in vitro* usually results from the use of peptides at a high concentration, generating a high level of specific

peptide/MHC complexes on antigen presenting cells (APCs), which will effectively activate these CTL (Alexander-Miller *et al.*, Proc Natl Acad Sci USA 93: 4102-7 (1996)).

SUMMARY OF THE INVENTION

In the present invention, the novel molecular targets were identified using genome-wide cDNA microarray analysis of cancer cells purified from HRPC tissues by means of LMM (laser microbeam microdissection). The assays identified a number of de-regulated genes in HRPCs, some of which are involved in androgen-independence and the aggressive phenotype. The present data provide a better understanding of the molecular mechanisms underlying clinical HRPCs, and provide genes whose products serve as molecular targets for treatment for HRPC.

To characterize the molecular features of clinical hormone-refractory prostate cancers (HRPCs), the present inventors analyzed gene-expression profiles of 25 clinical HRPCs and 10 hormone-sensitive prostate cancers (HSPCs) by genome-wide cDNA microarrays in combination with LMM. An unsupervised clustering analysis clearly distinguished expression patterns of HRPC cells from those of HSPC cells. In addition, primary and metastatic HRPCs from individual patients were closely clustered regardless of the kind of metastatic organs. A supervised clustering analysis identified 36 up-regulated genes and 70 down-regulated genes in HRPCs, compared with HSPCs ($P < 0.0001$, gap > 1.5). The present inventors observed over-expression of AR, ANLN, and SNRPE, and down-regulation of NR4A1, CYP27A1, and HLA-A antigen in HRPC. Such genes were considered to be related to the androgen-independent and more aggressive phenotype of HRPCs. In fact, knockdown of some over-expressing genes by siRNA resulted in drastic attenuation of prostate cancer cell growth. This precise microarray analysis of HRPC cells provides useful information for understanding the molecular mechanism of HRPC progression as well as for identifying molecular targets for development of treatment of HRPCs.

The present invention is based in part on the discovery of a pattern of gene expression correlated with HRPC and HSPC. The genes that are differentially expressed in HRPC compared to HSPC are collectively referred to herein as "HRPC-associated genes", "HRPC genes", "HRPC marker genes", "HRPC nucleic acids" or "HRPC polynucleotides" and the corresponding encoded polypeptides are referred to as "HRPC polypeptides" or "HRPC proteins".

Accordingly, the present invention features a method of diagnosing, testing, or identifying HRPC in a subject by determining an expression (*e.g.*, transcription or translation) level of an HRPC-associated gene in a patient-derived test cell population or a biological sample such as tissue sample (*e.g.*, serum or prostate tissue). Alternatively, HRPC cells in a test sample can be detected using an HRPC-associated gene as a tumor marker. By HRPC associated gene is meant a gene that is characterized by an expression level which differs in an HRPC cell compared to HSPC cell. An HRPC-associated gene includes, for example, HRPC 1-106. An alteration, *e.g.* increase or decrease of the level of expression of the gene compared to an expression level of the gene in HSPC, indicates that the subject has HRPC.

In the context of the present invention, the phrase "control level" refers to a protein or gene expression level detected in a control sample. The control level can be from a normal sample or a cancer sample. Therefore a control level can include a normal control level, an HSPC control level, and an HRPC control level and such. A control level can be a single expression pattern derived from a single reference population or an average or a standard value of expressions obtained from a plurality of reference populations. For example, the control level can be a database of expression patterns from previously tested cells. An "HRPC control level" and "HSPC control level" refers to an expression profile of HRPC-associated genes in a population suffering from HRPC and HSPC, respectively. Typically, an HSPC patient is defined as a good responder for androgen-ablation therapy. An expression pattern (levels) determined from a biopsy tissue sample comprising prostate cancer cells obtained from a responder for androgen-ablation therapy can be used as an HSPC control.

An increase in the expression level of one or more of HRPC 1-36 detected in a test sample as compared to a level in HSPC indicates that the subject (from which the sample was obtained) suffers from HRPC. In contrast, a decrease in the expression level of one or more HRPC 37-106 detected in a test sample as compared to a level in HSPC indicates that said subject suffers from HRPC.

Alternatively, expression of a panel of HRPC-associated genes in a sample can be compared to an HRPC control level of the same panel of genes. A similarity between a sample expression and an HRPC control expression indicates that the subject (from which the sample was obtained) suffers from HRPC. By HRPC control level is meant the expression profile of the panel of HRPC-associated genes found in a population suffering from HRPC.

According to the present invention, gene expression level is deemed "altered" when gene expression is increased or decreased 10% or more, 25% or more, or 50% or more, as compared to the level in a control (*e.g.* HSPC). Alternately, the gene expression is deemed altered if gene expression is increased or decreased 1, 2, 5 or more fold as compared to the level in a control (*e.g.* HSPC). Expression can be determined by method known in the art, for example, by detecting hybridization, *e.g.* on an array, of an HRPC-associated gene probe to a gene transcript of the patient-derived tissue sample.

In the context of the present invention, the patient derived tissue sample is any tissue obtained from a test subject, *e.g.* a patient known or suspected to have HRPC. For example, the tissue sample can be comprised of epithelial cells. More particularly, the tissue sample can comprise epithelial cells from prostate tissue.

The present invention provides methods for discriminating HRPC from HSPC and detecting HRPC cells with high sensitivity using HRPC 1-106.

The present invention also provides an HRPC reference expression profile, comprising a gene expression level of two or more of HRPC 1-106. Alternatively, the present invention provides an HRPC reference expression profile that comprises the levels of expression of two or more HRPC 1-36 or HRPC 37-106. In the context of the present invention, an "HRPC reference expression profile" includes a gene expression profile in HSPC cells, HRPC cells, or such.

The present invention further provides methods of identifying an agent that inhibits or enhances the expression or activity of an HRPC-associated gene, *e.g.* HRPC 1-106, by contacting a test cell expressing an HRPC-associated gene with a test agent and determining the expression level or activity of the HRPC-associated gene or the biological activity of its gene product. Biological activities of the protein encoded by the HRPC-associated gene include, for example, the promotion of cell proliferation.

The test cell can be an epithelial cell, for example, an epithelial cell obtained from prostate tissue. A decrease in the expression level of one or more HRPC-associated up-regulated genes or in the biological activity of its gene product in the presence of a test agent indicates that the test agent is an inhibitor of expression or function of the HRPC-associated gene and is useful to reduce a symptom of HRPC. An example of an HRPC-associated up-regulated gene includes HRPC 1-36. Alternatively, an increase in the expression level of one

or more HRPC-associated down-regulated genes or in the biological activity of its gene product in the presence of a test agent indicates that said test agent is an enhancer of expression or function of the HRPC associated gene and is useful to reduce a symptom of HRPC. An example of an HRPC-associated down-regulated gene includes HRPC 37-106.

The present invention also provides a kit comprising two or more detection reagents each of which binds to an HRPC polynucleotide or an HRPC polypeptide. Also provided is an array of nucleic acids, each of which nucleotide binds to an HRPC nucleic acid.

The lists of the genes associated with HRPC also provide information which is essential to establish novel chemo-preventive drugs effective for treating HRPC.

Therapeutic methods of the present invention include methods of treating or preventing HRPC in a subject including the step of administering to the subject an antisense composition. In the context of the present invention, the antisense composition reduces the expression of a specific target gene. For example, the antisense composition can contain an oligonucleotide or polynucleotide which is complementary to an HRPC-associated gene sequence selected from the group consisting of HRPC 1-36. Alternatively, the present methods can include the step of administering to a subject a small interfering RNA (siRNA) composition. In the context of the present invention, the siRNA composition reduces the expression of an HRPC nucleic acid selected from the group consisting of HRPC 1-36, *e.g.* ANLN (SEQ ID NO: 4 encoded by SEQ ID NO:3; GenBank Accession No. NM_018685) or SNRPE (SEQ ID NO: 2 encoded by SEQ ID NO:1; GenBank Accession No. NM_003094).

In yet another method, the treatment or prevention of HRPC in a subject can be carried out by administering to a subject a ribozyme composition. In the context of the present invention, a nucleic acid-specific ribozyme composition reduces the expression of an HRPC nucleic acid selected from the group consisting of HRPC 1-36. Other therapeutic methods include those in which a subject is administered with a compound that increases the expression of one or more of HRPC 37-106 or the activity of polypeptides encoded by one or more of HRPC 37-106. Furthermore, HRPC can be treated by administering a protein encoded by any one of HRPC 37-106. The protein can be directly administered to the patient or, alternatively, can be expressed *in vivo* subsequent to being introduced into the patient, for example, by administering an expression vector or host cell carrying the down-regulated

marker gene of interest. Suitable methods for *in vivo* expression of a gene of interest are known in the art.

The present invention also includes vaccines and vaccination methods. For example, a method of treating or preventing HRPC in a subject can involve administering to the subject a vaccine containing a polypeptide encoded by a nucleic acid selected from the group consisting of HRPC 1-36 or an immunologically active fragment of such a polypeptide. In the context of the present invention, an immunologically active fragment is a polypeptide that is shorter in length than the full-length naturally-occurring protein and which induces an immune response analogous to that induced by the full-length protein. For example, an immunologically active fragment should be at least eight-amino-acid-residue long and capable of stimulating an immune cell such as a T cell or a B cell. Immune cell stimulation can be measured by detecting cell proliferation, elaboration of cytokines (*e.g.* IL-2), or production of an antibody.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference herein in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

One advantage of the methods described herein is that prostate cancer is often identified prior to detection of overt clinical symptoms. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows a result of the laser microbeam microdissection (LMM). Normal prostatic epithelial cells (NP cells) and hormone refractory prostate cancer cells (HRPC cells) of bone metastasis were microdissected from H&E-stained sections to exclude the contamination of stromal cells and host organ cells at the metastatic site. Lane **A**, pre-microdissected tissue; Lane **B**, post-microdissected tissue; Lane **C**, microdissected cells.

RNAs were extracted from these microdissected cells (**C**) and after 2-round T7 amplification, they were applied to genome-wide cDNA microarrays.

Fig. 2 (**A**) shows the dendrogram of an unsupervised clustering analysis of 254 genes (vertical columns) across 35 prostate cancers (horizontal rows). The unsupervised clustering analysis clearly distinguished 25 HRPCs (red line) from 10 HSPCs (blue line). Small subsets of cluster constituted by metastatic HRPC cells (-B: bone metastasis, -L: lymph-node metastasis, -Li: liver metastasis) and HRPC cells at the primary site (-P: prostate) from the same patients are boxed. (**B**) is the dendrogram of a supervised clustering analysis of 106 genes (vertical columns) across 13 HRPCs at the prostate and 10 HSPCs (horizontal rows). Each cell in the matrix represents the expression level of a single transcript in a single sample. Red and green indicate transcript levels, above and below the median for that gene across all samples. Black represents unchanged expression and gray represents no detectable expression. The 36 up-regulated genes and 70 down-regulated genes that can distinguish HRPC cells from HSPC cells were listed in **Tables 1** and **2**, respectively.

Fig. 3 shows the result of semi-quantitative RT-PCR, confirming the elevated expression of eleven genes that could distinguish HRPC cells from HSPC cells (7 HRPCs and 7 HSPCs microdissected from prostrate cancer tissues). ACTB was used to quantify the each of cDNA contents.

Fig. 4 shows the result of an immunohistochemical analysis of prostate cancer tissues by anti-AR monoclonal antibody. Immunoreactivity with anti-AR antibody exhibited positive staining in the nucleus of HRPC cells (**A**), HSPC cells (**B**) and normal prostate epithelial (NP) cells (**C**). Their staining intensity or patterns in HRPC cells is similar to those in HSPC and NP cells, although the mRNA level of AR in HRPC cells was much higher that in HSPC and NP cells (**D**). (**D**) aslo shows that the expression levels of AR-regulated genes, PSA and NKX3.1, in HRPC cells were similar to those in HSPC and NP cells. ACTB was used to quantify the each of cDNA contents.

Fig. 5 shows that knockdown of ANLN and SNRPE transcripts by siRNA in PC cells attenuated their growth and viability. (**A**) and (**D**) shows knockdown effect of siRNA on ANLN and SNRPE in HRPC cell line 22Rv1, which was evaluated by semi-quantitative RT-PCR using cells transfected with each of siRNA-expressing vectors to ANLN (siANLN), SNRPE (si1, 3), and a negative control vector (siEGFP). ACTB was used to quantify RNAs.

Colony formation assay was performed on 22Rv1 cells (**B**, **E**) transfected with each of indicated siRNA-expressing vectors to ANLN (siANLN), SNRPE (si1, 3), and a negative control vector (siEGFP). Cells were visualized with 0.1% crystal violet staining after 14-day incubation with Geneticin. MTT assay was performed for each of 22Rv1 (**C**, **F**) transfected with indicated siRNA-expressing vectors to ANLN (siANLN), SNRPE (si1, 3), or a negative control vector (siEGFP). Y-axis in (**C**) and (**F**) means absorbance at 490 nm, and at 630 nm as reference, measured with a microplate reader. These experiments were carried out in triplicate and the average is plotted with error bars indicating SDs after 14-day incubation with Geneticin. * Means p value of <0.01 (Students' t-test).

DISCLOSURE OF THE INVENTION

The words "a", "an" and "the" as used herein mean "at least one" unless otherwise specifically indicated.

The present invention is based in part on a discovery of changes in expression patterns of multiple nucleic acids between cancerous cells of patients with hormone refractory prostate cancer (HRPC) and cancerous cells of patients with hormone sensitive prostate cancer (HSPC). These expression patterns were compared and differently expressed genes were identified using a comprehensive cDNA microarray system.

The differentially expressed genes identified herein are useful for diagnostic purposes as markers of HRPC or as gene targets, the expression of which is altered to treat or alleviate a symptom of HRPC. HRPC can be diagnosed by measuring the expression levels of HRPC-associated genes whose expression levels are altered in the transition from HSPC to HRPC.

Alternatively, the genes differentially expressed between HSPC and HRPC identified herein find diagnostic utility as markers for distinguishing HRPC from HSPC and as HRPC gene targets, the expression of which can be altered to treat or alleviate a symptom of HRPC.

The genes whose expression level is modulated (*i.e.*, increased or decreased) in HRPC patients are summarized in **Tables 1** and **2**, and are collectively referred to herein as "HRPC-associated genes", "HRPC genes", "HRPC marker genes", "HRPC nucleic acids" or "HRPC polynucleotides" and the corresponding encoded polypeptides are referred to as "HRPC polypeptides" or "HRPC proteins". Among the HRPC-associated genes, genes

whose expression level were increased or decreased in cells from HRPC patients as compared to that in cells from HSPC patients are referred to as "HRPC-associated up-regulated genes" and "HRPC-associated down-regulated genes", respectively. Unless otherwise indicated, "HRPC-associated genes" or an expression equivalent thereto refers to any of the sequences disclosed herein (*e.g.* HRPC 1-106). The genes that have been previously described are presented along with their database accession numbers.

By measuring expression of the various genes of the present invention in a sample of cells, HRPC can be diagnosed. Similarly, measuring the expression of these genes in response to various agents can identify agents for treating HRPC.

The present invention involves determining (*e.g.* measuring) the expression of at least one, and up to all of the HRPC-associated genes listed in **Tables 1** and **2**. Using sequence information provided by the GenBank® database entries for known sequences, the HRPC associated genes can be detected and measured using techniques well known to one of ordinary skill in the art. For example, sequences within the sequence database entries corresponding to HRPC associated genes can be used to construct probes for detecting RNA sequences corresponding to HRPC associated genes in, for example, Northern blot hybridization analyses. Probes typically include at least 10, at least 20, at least 50, at least 100, at least 200 contiguous nucleotides of a reference sequence. As another example, the sequences can be used to construct primers for specifically amplifying the HRPC nucleic acid in, for example, amplification-based detection methods such as reverse-transcription based polymerase chain reaction (RT-PCR).

The expression level(s) of one or more of HRPC-associated genes in a test cell population (*e.g.* a patient-derived tissue sample) is then compared to the expression level(s) of the same gene(s) in a reference population. The reference cell population includes one or more cells for which the compared parameter is known, and can be a population of HRPC cells or non-HRPC cells, for example, HSPC cells or non-transformed cells. The expression level(s) of HRPC 1-106 in the specimens from the test cell population and reference cell population can be determined at the same time. Alternatively, expression levels of HRPC 1-106 in reference cell population can be determined by a statistical method based on the results obtained by analyzing the expression level(s) of the gene(s) in specimens of previously collected prostate ductal carcinoma cells (*e.g.* HRPC cells or non-HRPC cells, including HSPC cells).

Whether or not a pattern of gene expression in a test cell population matches that of a reference cell population can be used as an indicator for judging an HRPC. For example, non-HRPC cells such as HSPC cells can be used as the reference cell population. When the expression level of the gene in a test cell population does not fall within the range (*i.e.*, is increased or decreased) of a HSPC reference cell population, the subject is judged to have HRPC. For example, increased expression levels (*e.g.*, transcription or translation) of one, two or more genes selected from HRPC 1-36 in the test cell population in comparison to a HSPC reference cell population is indicative of HRPC cells. Likewise, decreased expression levels of one, two or more genes selected from HRPC 37-106 in the test cell population in comparison to a HSPC reference cell population is indicative of HRPC cells.

Moreover, if the reference cell population is made up of HRPC cells, a similarity in gene expression profile between the test cell population and the reference cell population indicates that the test cell population includes HRPC cells. For example, substantially equivalent or similar expression levels of one, two or more genes selected from HRPC 1-106 in the test cell population in comparision to a HRPC reference cell population is indicative of HRPC cells.

A level of expression of an HRPC marker gene in a test cell population is considered "altered" if it varies from the expression level of the corresponding HRPC marker gene in a reference cell population by more than 1.1, more than 1.5, more than 2.0, more than 5.0, more than 10.0 or more fold. In some embodiments, a level of expression of an HRPC marker gene in a test cell population is considered "altered" (*i.e.*, increased or decreased) if it varies from the expression level of the corresponding HRPC marker gene in a reference cell population by 10%, 20%, 25%, 30%, 50%, 100%, 200%, or more.

Differential gene expression between a test cell population and a reference cell population can be normalized to a control nucleic acid, *e.g.* a housekeeping gene. For example, a control nucleic acid is one which is known not to differ depending on the cancerous or non-cancerous state of the cell. The expression level of a control nucleic acid in the test and reference population can be used to normalize signal levels in the test and reference populations. Exemplary control genes include, but are not limited to, β-actin, glyceraldehyde 3- phosphate dehydrogenase, and ribosomal protein P1.

The test cell population can be compared to multiple reference cell populations. Each of the multiple reference cell populations can differ in a known parameter. Thus, a test cell population can be compared to a first reference cell population known to contain *e.g.* HRPC cells, as well as a second reference population known to contain *e.g.* HSPC cells. The test cell population can include cells from a tissue known to or suspected to contain HRPC cells or a cell sample from a subject known to or suspected to suffer from HRPC.

The test cell population can be obtained from a bodily tissue or a bodily fluid, *e.g.* biological fluid (*e.g.*, blood, serum, plasma, urine or sputum). The test cell population can also be obtained from prostate tissue. The test cell population can comprise an epithelial cell, for example a prostate tissue epithelial cell. The epithelial cell can obtained from a tissue known or suspected to be cancerous, for example, cancerous prostate tissue and HSPC tissue. Cells in the reference cell population can be obtained from a bodily tissue or fluid type similar to that of the test cell population. Optionally, the reference cell population can be a cell line, *e.g.* an HRPC cell line (*i.e.* a positive control) or an HSPC cell line (*i.e.* a negative control). Alternatively, the gene expression levels of a reference (control) cell population can be determined based on a database of molecular information derived from cells for which the assayed parameter or condition whose state is known (*e.g.* being cancerous, HRPC, HSPC, or such)

The subject or patient is can be a mammal. Exemplary mammals include, but are not limited to, humans, non-human primates, mice, rats, dogs, cats, horses, and cows.

Expression of the genes disclosed herein can be determined at the protein or nucleic acid level using methods known in the art. For example, Northern hybridization analysis using probes which specifically recognize one or more of these nucleic acid sequences can be used to determine gene expression. Alternatively, gene expression can be measured using reverse-transcription-based PCR assays, *e.g.* using primers specific for the differentially expressed gene sequences. Expression can also be determined at the protein level, *i.e.* by measuring the level of translation of a polypeptide encoded by a gene described herein, or biological activity thereof. Such methods are well known in the art and include, but are not limited to, immunoassays that utilize antibodies to proteins encoded by the genes. The biological activities of the proteins encoded by the genes in Tables 1 and 2 are generally well known.

Diagnosing HRPC:

In the context of the present invention, HRPC is diagnosed by measuring the expression level of one or more HRPC polynucleotides in a test population of cells or a patient-derived biological sample. The methods of the present invention also include methods of testing for HRPC or identifying HRPC, and also include methods of discriminating HRPC from HSPC. The test cell population can be comprised of epithelial cells, *e.g.* epithelial cells obtained from prostate tissue. Particularly, the prostate tissue includes cancerous prostate tissue. Furthermore, the cancerous prostate tissue comprises HSPC. Gene expression can also be measured from blood, serum or plasma or other bodily fluids including urine. The biological samples also can be used for measuring protein levels. For example, the protein level in blood, serum, plasma or urine derived from a subject to be diagnosed can be measured by immunoassay or another conventional biological assay known in the art.

Expression level(s) of one or more of HRPC-associated genes, *e.g.* HRPC 1-106, is determined in the test cell population or biological sample and compared to expresson level(s) of one or more HRPC-associated gene(s) assayed in a control cell population or biological sample (*e.g.*, from a HSPC or HRPC cell population or a biological sample from a HSPC or HRPC patient).

An HSPC control level is an expression profile of one or more HRPC-associated genes typically found in a population known to be suffering from HSPC but not from HRPC. An HSPC control level can be determined, for example, by measuring protein or gene expression levels of one or more HRPC-associated genes/proteins in prostate cancer cells of HSPC patients. The HSPC control level can be a single expression profile obtained by determining expression levels of one or more HRPC-associated genes in a cell population or a biological sample obtained from an HSPC patient. Alternatively, the HSPC control level can be an average or a standard value of expression levels of the above-mentioned genes in a cell population or a biological sample obtained from a plurality of HSPC patients. The cell populations or biological samples for determining the HSPC control level can be collected from patients suffering from HSPC but not from HRPC (*i.e.*, HSPC patients), after an androgen-ablation therapy. HSPC patients can be defined as those patients who respond well to the androgen ablation therapy.

An HRPC control level is an expression profile of one or more HRPC-associated genes typically found in a population known to be suffering from HRPC. The HRPC control level can be a single expression profile obtained by determining expression levels of one or more HRPC-associated genes in a cell population or a biological sample obtained from an HRPC patient. Alternatively, the HRPC control level can be an average or a standard value of expression levels of the above-mentioned genes in a cell population or a biological sample obtained from a plurality of HRPC patients. The cell populations or biological samples for determining the HRPC control level can be collected from patients suffering from HRPC, who has become less or not responsive to the androgen ablation therapy.

An alteration, *e.g.* an increase or a decrease, in the level of expression of one or more HRPC-associated genes in the patient-derived test cell population or biological sample indicates that the subject is suffering from HRPC. For example, an increase in the expression of one or more up-regulated HRPC-associated genes, HRPC 1-36, in the test population or the biological sample as compared to the expression level in HSPC indicates that the subject is suffering from HRPC. Conversely, a decrease in expression of one or more down-regulated HRPC-associated genes, HRPC 37-106, in the test population or biological sample compared to the expression level in HSPC indicates that the subject is suffering from or HRPC.

Alteration of one or more of the HRPC-associated genes in the test population as compared to the expression level in HSPC indicates that the subject suffers from HRPC. For example, alteration of at least 1%, at least 5%, at least 25%, at least 50%, at least 60%, at least 80%, at least 90% or more of the panel of HRPC-associated genes (HRPC 1-36 or HRPC 37-106) indicates that the subject suffers from HRPC.

In the present invention, a standard value of the level of HRPC-associated gene can be determined statistically. For example, expression levels of HRPC-associated genes in a cell population or biological sample obtained from non-HRPC patients (*e.g.* HSPC patients) can be measured to determine the standard levels of the HRPC-associated genes statistically. When a statistically sufficient population is gathered, a value in the range of twice or three times the standard deviation (S.D.) from the mean value can be used as the standard value. Therefore, values corresponding to the mean value + 2 x S.D. or mean value + 3 x S.D. can be used as standard values. The standard values set as described theoretically comprise 90% and 99.7% of HSPC patients, respectively.

Alternatively, standard values can also be set based on the actual expression levels of HRPC-associated genes in HRPC patients. Generally, standard values set this way minimize the percentage of false positives, and are selected from a range of values satisfying conditions that can maximize detection sensitivity. Herein, the percentage of false positives refers to a percentage, among non-HRPC patients, of patients (i) whose levels of HRPC-associated up-regulated genes are judged to be higher than a standard value, or (ii) whose levels of HRPC-associated down-regulated genes are judged to be lower than a standard value. On the contrary, the percentage, among non-HRPC patients, of patients (i) whose levels of HRPC-associated up-regulated genes are judged to be lower than a standard value or (ii) whose levels of HRPC-associated down-regulated genes are judged to be higher than a standard value indicates specificity. That is, the sum of the false positive percentage and the specificity is always 1. The detection sensitivity refers to the percentage of HRPC patients (i) whose levels of HRPC-associated up-regulated genes are judged to be higher than a standard value or (ii) whose levels of HRPC-associated down-regulated genes are judged to be lower than a standard value, among all HRPC patients within a population of individuals for whom the presence of HRPC has been determined.

Alternatively, according to the present invention, an intermediate result for examining the condition of a subject can be provided. Such intermediate result can be combined with additional information to assist a doctor, nurse, or other practitioner to determine that a subject suffers from HRPC. Further, the present invention relates to a method for screening a person who is required to be further diagnosed for HRPC. After screening for HRPC, persons indicating positive result are recommended to be submitted further screening test, or medical treatment to confirm whether they truly suffer from HRPC. Accordingly, the present invention also provides proteins encoded by HRPC-associated genes as blood tumor markers for diagnosing or screening of HRPC.

Alternatively, the present invention can be used to detect cancerous cells in a subject-derived tissue, and providing a doctor with information for determining whether the subject suffers from HRPC. Accordingly, the present invention involves determining (*e.g.*, measuring) the level of HRPC-associated genes in subject derived samples, for example, fluid or tissue samples, including blood, serum, plasma, urine or prostate tissue. In the present invention, a method for diagnosing HRPC also includes a method for testing or detecting HRPC. Alternatively, in the present invention, diagnosing HRPC also refers to showing a suspicion, risk, or possibility of HRPC in a subject.

Further, HRPC cells in a test sample collected from a subject to be diagnosed can be detected using HRPC-associated genes. Accordingly, the present invention also provides a method of detecting hormone-refractory prostate cancer (HRPC) cells in a test sample collected from a patient, comprising determining a level of expression of one or more HRPC-associated genes in a patient-derived biological sample comprising cells, wherein an increase or decrease of said expression level compared to the expression level of said genes in hormone-sensitive prostate cancer (HSPC) indicates that hormone-refractory prostate cancer cells are detected in said test sample. In some embodiments, the test sample is a solid tissue sample, for example, a biopsy tissue, for example, from prostate tissue.

The expression levels of the HRPC 1-106 in a particular biological sample specimen can be estimated by quantifying mRNA corresponding to or protein encoded by HRPC 1-106. Quantification methods for mRNA are known to those skilled in the art. For example, the levels of mRNAs corresponding to HRPC 1-106 can be estimated by Northern blotting or RT-PCR. In some embodiments, mRNA expression levels are quantified by real-time and/or quantitative PCR. Since the nucleotide sequence of HRPC 1-106 have already been reported, anyone skilled in the art can design the nucleotide sequences for probes or primers to quantify HRPC 1-106.

Also, the expression level of HRPC 1-106 can be analyzed based on the activity or quantity of protein encoded by the gene. A method for determining the quantity of the HRPC 1-106 protein is shown below. For example, immunoassay methods are useful for the determination of proteins in biological materials. Any biological materials can be used for the determination of the protein or its activity. For example, a blood sample (*e.g.*, whole blood, serum or plasma) is analyzed for estimation of the protein encoded by a serum marker. On the other hand, a suitable method can be selected for the determination of the activity of a protein encoded by HRPC 1-106 according to the activity of each protein to be analyzed.

In the present invention, a diagnostic agent for diagnosing HRPC is also provided. The diagnostic agent of the present invention comprises a compound that binds to a polynucleotide or a polypeptide of the present invention. An oligonucleotide that hybridizes to the polynucleotide of HRPC 1-106, or an antibody or fragment thereof that binds to the polypeptide of HRPC 1-106 can be used as such a compound.

In the present invention, HRPC to be diagnosed or tested is defined as PC having less

or no responsiveness to hormone therapy such as androgen-ablation therapy. For instance, clinical HRPC can be defined by elevation of serum PSA levels at three consecutive times and/or enlargement of tumor in spite of androgen-ablation therapy. In the present invention, PC other than HRPC can be defined as HSPC.

Identifying Agents that inhibit or enhance HRPC-associated gene expression:

An agent that inhibits the expression or activity of an HRPC-associated gene or the activity of its gene product can be identified by contacting a test cell population expressing an HRPC-associated up-regulated gene with a test agent and then determining the expression level or activity of the HRPC-associated gene. A decrease in the level of expression or activity of the HRPC-associated up-regulated gene or in the level of activity of its gene product in the presence of the agent as compared to the expression or activity in the absence of the test agent indicates that the agent is an inhibitor of an HRPC associated up-regulated gene and useful in inhibiting HRPC. Exemplified HRPC-associated up-regulated genes include HRPC 1-36.

Alternatively, an agent that enhances the expression of an HRPC-associated down-regulated gene or the activity of its gene product can be identified by contacting a test cell population expressing an HRPC associated down-regulated gene with a test agent and then determining the expression level or activity of the HRPC-associated down-regulated gene. An increase in the level of expression of the HRPC-associated down-regulated gene or in the level of activity of its gene products as compared to the expression or activity in the absence of the test agent indicates that the test agent augments expression of HRPC-associated down-regulated gene or activity of its gene product. Exemplified HRPC-associated down-regulated genes include HRPC 37-106.

The test cell population can be comprised of any cells expressing the HRPC-associated genes. For example, the test cell population can contain epithelial cells, such as epithelial cells derived from prostate tissue. Alternatively, the test cell population can contain hormone-refractory prostate cancer cells. Furthermore, the test cell can be an immortalized cell line derived from an HRPC cell. Alternatively, the test cell population can be cells which have been transfected with an HRPC-associated gene or which have been transfected with a regulatory sequence (*e.g.* promoter sequence) from an HRPC-associated gene operably linked to a reporter gene. If the expression of the reporter gene or the activity of an HRPC-associated gene product is increased in the presence of a test agent as compared to the

expression or activity in the absence of the test agent, the test agent is judged as enhancing the expression or activity of the HRPC-associated gene. Conversely, if the expression of the reporter gene or the activity of the HRPC-associated gene product is decreased in the presence of a test agent as compared to the expression or activity in the absence of the test agent, the test agent is judged as inhibiting the espression or activity of the HRPC-associated gene.

Assessing efficacy of treatment of HRPC in a subject:

The differentially expressed HRPC-associated genes identified herein also allow for the course of treatment of HRPC to be monitored. In this method, a test cell population is provided from a subject undergoing treatment for HRPC. If desired, test cell populations are obtained from the subject at various time points, before, during, and/or after treatment. Expression of one or more of the HRPC-associated genes in the cell population is then determined and compared to a reference cell population which includes cells whose PC state is known (*e.g.*, HSPC cell population).

If the reference cell population contains no HRPC cells, a similarity in the expression of an HRPC-associated gene in the test cell population and the reference cell population indicates that the treatment of interest is efficacious. However, a difference in the expression of an HRPC-associated gene in the test population in comparison to a HSPC reference cell population indicates a less favorable clinical outcome or prognosis. Similarly, if the reference cell population is comprised of HRPC cells, a difference between the expression of an HRPC-associated gene in the test cell population and the reference cell population indicates that the treatment of interest is efficacious, while a similarity in the expression of an HRPC-associated gene in the test population and the reference cell population indicates a less favorable clinical outcome or prognosis.

Additionally, the expression level of one or more HRPC-associated genes determined in a subject-derived biological sample obtained after treatment (*i.e.* post-treatment levels) can be compared to the expression level of the one or more HRPC-associated genes determined in a subject-derived biological sample obtained prior to treatment onset (*i.e.* pre-treatment levels). If the HRPC-associated gene is an up-regulated gene, a decrease in the expression level in a post-treatment sample indicates that the treatment of interest is efficacious while an increase or maintenance in the expression level in the post-treatment sample indicates a less favorable clinical outcome or prognosis. Conversely, if the HRPC-associated gene is a down-regulated gene, an increase in the expression level in a post-treatment sample indicates that

the treatment of interest is efficacious while a decrease or maintenance in the expression level in the post-treatment sample indicates a less favorable clinical outcome or prognosis.

As used herein, the term "efficacious" indicates that the treatment leads to a reduction in the expression of a pathologically up-regulated gene, an increase in the expression of a pathologically down-regulated gene, or a decrease in size, prevalence, or metastatic potential of HRPC in a subject. When a treatment of interest is applied prophylactically, the term "efficacious" means that the treatment retards or prevents an HRPC from forming or retards, prevents, or alleviates a symptom of clinical HRPC. Assessment of prostate tumors can be made using standard clinical protocols.

Treating or ameliorating HRPC or prevention of the onset of HRPC includes any of the following steps, such as surgical removal of HRPC cells, inhibition of the growth of cancerous prostate cells, involution or regression of a prostate tumor, induction of remission and suppression of occurrence of prostate cancer and enhancement of sensitivity for the androgen-ablation therapy. Effectively treating HRPC decreases mortality and improves the prognosis of individuals having HRPC, decreases the levels of tumor markers in the blood, and alleviates detectable symptoms accompanying HRPC.

In addition, efficaciousness can be determined in association with any known method for diagnosing or treating HRPC. HRPC can be diagnosed, for example, by identifying symptomatic anomalies, *e.g.* weight loss, abdominal pain, back pain, anorexia, nausea, vomiting and generalized malaise, weakness, and jaundice.

Selecting a therapeutic agent for treating HRPC that is appropriate for a particular individual:

Differences in the genetic makeup of individuals can result in differences in their relative abilities to metabolize various drugs. An agent that is metabolized in a subject to act as an anti-HRPC agent can manifest itself by inducing a change in a gene expression pattern in the subject's cells from that characteristic of a cancerous state to a gene expression pattern characteristic of a non-cancerous state. Accordingly, the differentially expressed HRPC-associated genes disclosed herein allow for candidate therapeutic or prophylactic inhibitors of HRPC to be tested in a test cell population from a selected subject in order to determine if the agent is a suitable inhibitor of HRPC in the subject.

To identify an inhibitor of HRPC appropriate for a specific subject, a test cell population from the subject is exposed to a therapeutic agent, and the expression of one or more of HRPC 1-106 genes is determined.

In the context of the method of the present invention, the test cell population contains an HRPC cell expressing an HRPC-associated gene. The test cell population can comprise epithelial cells. For example, a test cell population can be incubated in the presence of a candidate agent and the pattern of gene expression of the test sample can be measured and compared to one or more reference profiles, *e.g.* an HSPC or HRPC reference expression profile.

A decrease in expression of one or more of HRPC 1-36 or an increase in expression of one or more of HRPC 37-106 in a test cell population relative to a reference cell population containing HRPC indicates that the agent finds therapeutic use.

In the context of the present invention, the test agent can be any compound or composition. For example, the test agent can be a nucleic acid, a polypeptide, or a small organic compound. Exemplary, the test agents include, but are not limited to, immunomodulatory agents.

Screening assays for identifying therapeutic agents:

The differentially expressed HRPC-associated genes disclosed herein can also be used to identify candidate therapeutic agents for treating HRPC. The methods of the present invention involve screening a candidate therapeutic agents to determine if an agent can convert an expression profile of one or more HRPC-associated genes, such as HRPC 1-106 (*e.g.* TMEM46) characteristic of an HRPC state to a gene expression pattern characteristic of an HSPC state.

In the present invention, HRPC 1-106 are useful for screening therapeutic agents for treating or preventing HRPC.

In the instant method, a cell is exposed to a test agent or a plurality of test agents (sequentially or in combination) and the expression of one or more HRPC 1-106 in the cell is measured. The expression profile of the HRPC-associated gene(s) assayed in the test population is compared to expression level of the same HRPC-associated gene(s) in a reference cell population that is not exposed to the test agent.

An agent capable of stimulating the expression of an HRPC-associated down-regulated gene or suppressing the expression of an HRPC-associated up-regulated gene has clinical benefit. Such agents can be further tested for the ability to prevent HRPC in animals or test subjects.

In a further embodiment, the present invention provides methods for screening candidate agents for use in the treatment of HRPC. As discussed in detail above, by controlling the expression levels of HRPC marker genes or the activities of their gene products, one can control the onset and progression of HRPC. Thus, candidate agents for use in the treatment of HRPC can be identified through screening methods that use such expression levels and activities of HRPC marker genes as indices of the cancerous or non-cancerous state. In the context of the present invention, such screening can comprise, for example, the following steps:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of HRPC 1-106,

b) detecting the binding activity between the polypeptide and the test compound; and

c) selecting the test compound that binds to the polypeptide.

In preferred embodiment, a test compound selected by the method of the present invention may be candidate for further screening to evaluate the therapeutic effect thereof. That is, the above screening method further comprises the following steps;

d) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of HRPC 1-106,

e) detecting the biological activity of the polypeptide of step d), and

f) selecting a compound that suppresses the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of HRPC 1-36 as compared to the biological activity detected in the absence of the test compound, or enhances the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of HRPC 37-106 as compared to the biological activity detected in the absence of the test compound.

Alternatively, the above screening method further comprises the following steps;

d') contacting the test compound selected in step c) with test cells whose proliferation were enhanced by expressing HRPC 1-36 or suppressing HRPC 37-106,

e') detecting a cell proliferation of the test cells of step d'), and

f') selecting a compound that suppresses the cell proliferation as compared to the cell proliferation detected in the absence of the test compound.

Alternatively, the screening method of the present invention can comprise the following steps:

a) contacting a candidate compound with a cell expressing one or more marker genes, wherein the one or more marker genes are selected from the group consisting of HRPC 1-106; and

b) selecting the candidate compound that reduces the expression level of one or more marker genes selected from the group consisting of HRPC 1-36, or that elevates the expression level of one or more marker genes selected from the group consisting of HRPC 37-106.

In preferred embodiment, a candidate compound selected by the method of the present invention may be candidate for further screening to evaluate the therapeutic effect thereof. That is, the above screening method further comprises the following steps;

c) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of HRPC 1-106,

d) detecting the biological activity of the polypeptide of step c), and

e) selecting a compound that suppresses the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of HRPC 1-36 as compared to the biological activity detected in the absence of the candidate compound, or enhances the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of HRPC 37-106 as compared to the biological activity detected in the absence of the candidate compound.

Alternatively, the above screening method further comprises the following steps;

c') contacting the test compound selected in step b) with test cells whose proliferation were enhanced by expressing HRPC 1-36 or suppressing HRPC 37-106,

d') detecting a cell proliferation of the test cells of step c'), and

e') selecting a compound that suppresses the cell proliferation as compared to the cell proliferation detected in the absence of the candidate compound.

Cells expressing a marker gene include, for example, cell lines established from HRPC; such cells can be used for the above screening of the present invention.

Alternatively, the screening method of the present invention can comprise the following steps:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of HRPC 1-106;

b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of HRPC 1-36 as compared to the biological activity detected in the absence of the test compound, or enhances the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of HRPC 37-106 as compared to the biological activity detected in the absence of the test compound.

A protein for use in the screening method of the present invention can be obtained as a recombinant protein using the nucleotide sequence of the marker gene. Based on the information regarding the marker gene and its encoded protein, one skilled in the art can select any biological activity of the protein as an index for screening and any suitable measurement method to assay for the selected biological activity. For example, the biological activity is the promotion of cell proliferation, kinase activity, phosphatase activity, membrane transport, ubiquitination activity, metyltransferase activity or transcription activity.

Alternatively, the screening method of the present invention can comprise the following steps:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of one or more marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the one or more marker genes are selected from the group consisting of HRPC 1-106

b) measuring the expression level or activity of said reporter gene; and

c) selecting the candidate compound that reduces the expression or activity level of said reporter gene when said marker gene is an up-regulated marker gene selected from the group consisting of HRPC 1-36 as compared to a level in control, or that enhances the

expression level of said reporter gene when said marker gene is a down-regulated marker gene selected from the group consisting of HRPC 37-106, as compared to a level in control.

Suitable reporter genes and host cells are well known in the art. A reporter construct suitable for the screening method of the present invention can be prepared by using the transcriptional regulatory region of an HRPC marker gene. When the transcriptional regulatory region of the marker gene is known to those skilled in the art, a reporter construct can be prepared by using the previously known sequence information. When the transcriptional regulatory region of the marker gene remains unidentified, a nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library based on the nucleotide sequence information of the marker gene. In the present method, for example, a level detected in the absence of the test compound can be used as the control expression level to be compared.

The transcriptional regulatory region can be, for example, the promoter sequence of the HRPC-associated genes. The reporter construct required for the screening can be prepared by connecting reporter gene sequence to the transcriptional regulatory region of HRPC-associated genes. The transcriptional regulatory region of HRPC-associated genes herein is the region from start codon to at least 500bp upstream, for example, 1000bp, 5000bp or 10000bp upstream. A nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library or can be propagated by PCR. Methods for identifying a transcriptional regulatory region, and also assay protocol are well known (Molecular Cloning third edition chapter 17, 2001, Cold Springs Harbor Laboratory Press).

Plural screening methods can be combined. For example first step is a screening method to select the test agents that bind to HRPC-associated genes or proteins, or inhibit the expression of the genes, and next step is a screening method to select the test agents that alter the biological activity of HRPC-associated genes.

A compound isolated by the screening serves as a candidate for the development of drugs that inhibit or enhance the activity of the protein encoded by marker gene and can be applied to the treatment or prevention of HRPC.

Moreover, compounds in which a part of the structure of the compound inhibiting or enhancing the activity of proteins encoded by marker genes is converted by addition, deletion

and/or replacement are also included as the compounds obtainable by the screening methods of the present invention.

When administrating a compound isolated by the method of the present invention as a pharmaceutical for humans and other mammals, such as mice, rats, guinea-pigs, rabbits, cats, dogs, sheep, pigs, cattle, monkeys, baboons, and chimpanzees, the isolated compound can be directly administered or can be formulated into a dosage form using known pharmaceutical preparation methods. For example, according to the need, the drugs can be taken orally, as sugar-coated tablets, capsules, elixirs and microcapsules, or non-orally, in the form of injections of sterile solutions or suspensions with water or any other pharmaceutically acceptable liquid. For example, the compounds can be mixed with pharmaceutically acceptable carriers or media, specifically, sterilized water, physiological saline, plant-oils, emulsifiers, suspending agents, surfactants, stabilizers, flavoring agents, excipients, vehicles, preservatives, binders, and such, in a unit dose form required for generally accepted drug implementation. The amount of active ingredient contained in such a preparation makes a suitable dosage within the indicated range acquirable.

Examples of additives that can be admixed into tablets and capsules include, but are not limited to, binders, such as gelatin, corn starch, tragacanth gum and arabic gum; excipients, such as crystalline cellulose; swelling agents, such as corn starch, gelatin and alginic acid; lubricants, such as magnesium stearate; sweeteners, such as sucrose, lactose or saccharin; and flavoring agents, such as peppermint, Gaultheria adenothrix oil and cherry. When the unit-dose form is a capsule, a liquid carrier, such as an oil, can be further included in the above ingredients. Sterile composites for injection can be formulated following normal drug implementations using vehicles such as distilled water suitable for injection.

Physiological saline, glucose, and other isotonic liquids including adjuvants, such as D-sorbitol, D-mannnose, D-mannitol, and sodium chloride, can be used as aqueous solutions for injection. These can be used in conjunction with suitable solubilizers, such as alcohol, for example ethanol; polyalcohols, such as propylene glycol; and polyethylene glycol; and non-ionic surfactants, such as Polysorbate 80™ and HCO-50.

Sesame oil or soy-bean oil can be used as an oleaginous liquid, can be used in conjunction with benzyl benzoate or benzyl alcohol as a solubilizer and can be formulated with a buffer, such as phosphate buffer and sodium acetate buffer; a pain-killer, such as

procaine hydrochloride; a stabilizer, such as benzyl alcohol and phenol; and/or an anti-oxidant. A prepared injection can be filled into a suitable ampoule.

Methods well known to those skilled in the art can be used to administer the pharmaceutical composition of the present invention to patients, for example as an intraarterial, intravenous, or percutaneous injection or as an intranasal, transbronchial, intramuscular or oral administration. The dosage and method of administration vary according to the body-weight and age of a patient and the administration method; however, one skilled in the art can routinely select a suitable method of administration. If said compound is encodable by a DNA, the DNA can be inserted into a vector for gene therapy and the vector administered to a patient to perform the therapy. The dosage and method of administration vary according to the body-weight, age, and symptoms of the patient; however one skilled in the art can suitably select them.

For example, although the dose of a compound that binds to a protein of the present invention and regulates its activity depends on the symptoms, the dose is generally about 0.1 mg to about 100 mg per day, for example, about 1.0 mg to about 50 mg per day, for example, about 1.0 mg to about 20 mg per day, when administered orally to a normal adult human (weight 60 kg).

When administering the compound parenterally, in the form of an injection to a normal adult human (weight 60 kg), although there are some differences according to the patient, target organ, symptoms and method of administration, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg per day, for example, about 0.1 to about 20 mg per day, for example, about 0.1 to about 10 mg per day. In the case of other animals, the appropriate dosage amount can be routinely calculated by converting to 60 kgs of body-weight.

Kits:

The present invention also includes HRPC-detection reagents, for example, (i) nucleic acids each of which specifically binds to or identifies an HRPC nucleic acid, such as oligonucleotide sequences which are complementary to a portion of any one of HRPC nucleic acids (*e.g.*, HRPC 1-106), and (ii) antibodies each of which binds to a protein encoded by any one of HRPC nucleic acids (*e.g.*, HRPC 1-106). The detection reagents can be packaged together in the form of a kit. The reagents are packaged in separate containers, *e.g.* a nucleic

acid or antibody (either bound to a solid matrix or packaged separately with reagents for binding them to the matrix), a control reagent (positive and/or negative), and/or a detectable label. Instructions (*e.g.* written, tape, VCR, CD-ROM, *etc.*) for carrying out the assay can also be included in the kit. In some embodiments, the assay format of the kit is Northern hybridization or sandwich ELISA, both of which are known in the art.

For example, one or more HRPC detection reagents can be immobilized on a solid matrix, for example, a porous strip to form at least one HRPC detection site. The measurement or detection region of the porous strip can include a plurality of sites, each containing a nucleic acid. A test strip can also contain sites for negative and/or positive controls. Alternatively, control sites can be located on a separate strip from the test strip. Optionally, the different detection sites can contain different amounts of immobilized nucleic acids, *i.e.* a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test sample, the number of sites displaying a detectable signal provides a quantitative indication of the amount of HRPC nucleic acids present in the sample. The detection sites can be configured in any suitably detectable shape and are typically in the shape of a bar or dot spanning the width of a test strip.

Alternatively, the kit can contain a nucleic acid substrate array comprising one or more nucleic acids. The nucleic acids on the array specifically identify one or more nucleic acids sequences represented by HRPC 1-106. The expression of 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 40, or 50 or more of the nucleic acids represented by HRPC 1-106 are identified by virtue of the level of binding to an array test strip or chip. The substrate array can be on, *e.g.* a solid substrate, such as a "chip" described in U.S. Patent No.5,744,305, the contents of which are incorporated by reference herein in its entirety.

Arrays and pluralities:

The present invention also includes a nucleic acid substrate array comprising one or more nucleic acids. The nucleic acids on the array specifically correspond to one or more nucleic acid sequences represented by HRPC 1-106. The level of expression of 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 40, or 50 or more of the nucleic acids represented by HRPC 1-106 can be identified by detecting nucleic acid binding to the array.

The present invention also includes an isolated plurality (*i.e.* a mixture of two or more nucleic acids) of nucleic acids. The nucleic acids can be in a liquid phase or a solid

phase, *e.g.* immobilized on a solid support, for example, a chip or a nitrocellulose membrane. The plurality includes two or more of the nucleic acids represented by HRPC 1-106. In various embodiments, the plurality includes 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 40, or 50 or more of the nucleic acids represented by HRPC 1-106.

Methods of inhibiting HRPC:

The present invention further provides a method for treating or alleviating a symptom of HRPC in a subject by decreasing the expression or activity of one or more of the HRPC 1-36 (or the activity of its gene product) or increasing expression or activity of HRPC 37-106 (or the activity of its gene product). Suitable therapeutic compounds can be administered prophylactically or therapeutically to a subject suffering from (or susceptible of having) HRPC. Such subjects can be identified using standard clinical methods or by detecting an aberrant level of expression of one or more of the HRPC 1-106 or aberrant activity of a gene product of HRPC 1-106. In the context of the present invention, suitable therapeutic agents include, for example, inhibitors of cell cycle regulation, cell proliferation, and protein kinase activity.

The therapeutic methods of the present invention include the step of increasing the expression, function, or both of one or more gene products of genes whose expression is decreased ("down-regulated" or "under-expressed" genes) in HRPC cell relative to HSPC cells of the same tissue type from which the HRPC or HSPC cells are derived. In these methods, the subject is treated with an effective amount of a compound that increases the amount of one or more of the under-expressed (down-regulated) genes in the subject. Administration can be systemic or local. Suitable therapeutic compounds include a polypeptide product of an under-expressed gene, a biologically active fragment thereof, and a nucleic acid encoding an under-expressed gene and having expression control elements permitting expression in the HRPC cells. In addition, an agent that increases the level of expression of such a gene endogenous to the HRPC cells (*i.e.* which up-regulates the expression of the under-expressed gene or genes) is also included. Administration of such compounds counters the effects of aberrantly under-expressed gene or genes in the subject's prostate cells and improves the clinical condition of the subject.

Alternatively, the therapeutic method of the present invention can include the step of decreasing the expression, function, or both, of one or more gene products of genes whose expression is aberrantly increased ("up-regulated" or "over-expressed" gene) in prostate cells.

Expression can be inhibited in any of several ways known in the art. For example, expression can be inhibited by administering to the subject a nucleic acid that inhibits, or antagonizes, the expression of the over-expressed gene or genes, *e.g.* an antisense oligonucleotide or small interfering RNA which disrupts expression of the over-expressed gene or genes.

The term "specifically inhibit" in the context of inhibitory polynucleotides and polypeptides refers to the ability of an agent or ligand to inhibit the expression or the biological function of HRPC-associated genes and proteins encoded thereby. Specific inhibition typically results in at least about a 2-fold inhibition over background, for example, greater than about 10-fold or greater than 100-fold inhibition of HRPC-associated genes (*e.g.*, transcription or translation) or measured biological function (*e.g.*, cell growth or proliferation, inhibition of apoptosis, intracellular signaling from HRPC-associated genes). Expression levels and/or biological function can be measured in the context of comparing treated and untreated cells, or a cell population before and after treatment. In some embodiments, the expression or biological function of HRPC-associated genes and proteins encoded thereby is completely inhibited. Typically, specific inhibition is a statistically meaningful reduction in HRPC-associated genes expression or biological function (*e.g.*, $p \leq 0.05$) using an appropriate statistical test.

Antisense Nucleic Acids:

As noted above, antisense nucleic acids corresponding to the nucleotide sequence of HRPC 1-36 can be used to reduce the expression level of the HRPC 1-36. Antisense nucleic acids corresponding to HRPC 1-36 that are up-regulated in HRPC are useful for the treatment of HRPC. Specifically, the antisense nucleic acids of the present invention can act by binding to the HRPC 1-36 or mRNAs corresponding thereto, thereby inhibiting the transcription or translation of the genes, promoting the degradation of the mRNAs, and/or inhibiting the expression of proteins encoded by a nucleic acid selected from the group consisting of the HRPC 1-36, finally inhibiting the function of the proteins. The term "antisense nucleic acids" as used herein encompasses both nucleotides that are entirely complementary to the target sequence and those having a mismatch of one or more nucleotides, so long as the antisense nucleic acids can specifically hybridize to the target sequences. For example, the antisense nucleic acids of the present invention include polynucleotides that have a homology of at least 70% or higher, for example, at least 80%, 90% or higher, for example at least 95%, 97%, 99% or higher, over a span of at least 15 continuous nucleotides. Algorithms known in the art can

be used to determine the homology.

The antisense nucleic acid derivatives of the present invention act on cells producing the proteins encoded by marker genes by binding to the DNAs or mRNAs encoding the proteins, inhibiting their transcription or translation, promoting the degradation of the mRNAs, and inhibiting the expression of the proteins, thereby resulting in the inhibition of the protein function.

An antisense nucleic acid derivative of the present invention can be made into an external preparation, such as a liniment or a poultice, by admixing it with a suitable base material which is inactive against the nucleic acid.

Also, as needed, the antisense nucleic acids of the present invention can be formulated into tablets, powders, granules, capsules, liposome capsules, injections, solutions, nose-drops and freeze-drying agents by adding excipients, isotonic agents, solubilizers, stabilizers, preservatives, pain-killers, and such. These can be prepared by following known methods.

The antisense nucleic acids derivative of the present invention can be given to the patient by direct application onto the ailing site or by injection into a blood vessel so that it will reach the site of ailment. An antisense-mounting medium can also be used to increase durability and membrane-permeability. Examples include, but are not limited to, liposomes, poly-L-lysine, lipids, cholesterol, lipofectin or derivatives of these.

The dosage of the antisense nucleic acid derivative of the present invention can be adjusted suitably according to the patient's condition and used in desired amounts. For example, a dose range of 0.1 to 100 mg/kg, for example, 0.1 to 50 mg/kg can be administered.

The antisense nucleic acids of the present invention inhibit the expression of a protein of the present invention and are thereby useful for suppressing the biological activity of the protein of the invention. In addition, expression-inhibitors, comprising antisense nucleic acids of the present invention, are useful in that they can inhibit the biological activity of a protein of the present invention.

The antisense nucleic acids of present invention include modified oligonucleotides. For example, thioated oligonucleotides can be used to confer nuclease resistance to an oligonucleotide.

Alternatively, according to the present invention, use of antisense nucleic acids against a polynucleotide select from the group consisting of HRPC 1-36 for manufacturing a pharmaceutical composition for treating or preventing hormone-refractory prostate cancer is provided. Further, the present invention also provides antisense nucleic acids against a polynucleotide select from the group consisting of HRPC 1-36 for treating or preventing hormone-refractory prostate cancer.

siRNA:

Also, an siRNA against an HRPC marker gene can be used to reduce the expression level of the marker gene. Herein term "siRNA" refers to a double stranded RNA molecule which prevents translation of a target mRNA. Standard techniques for introducing siRNA into the cell can be used, including those in which DNA is a template from which RNA is transcribed. In the context of the present invention, the siRNA comprises a sense nucleic acid sequence and an anti-sense nucleic acid sequence against an up-regulated marker gene, such as HRPC 1-36. The siRNA is constructed such that a single transcript has both the sense and complementary antisense sequences from the target gene, *e.g.* a hairpin, which, in some embodiments, leads to production of micro RNA (miRNA). The siRNA can either be a dsRNA or shRNA.

As used herein, the term "dsRNA" refers to a construct of two RNA molecules comprising complementary sequences to one another and that have annealed together via the complementary sequences to form a double-stranded RNA molecule. The nucleotide sequence of two strands can comprise not only the "sense" or "antisense" RNAs selected from a protein coding sequence of target gene sequence, but also RNA molecule having a nucleotide sequence selected from non-coding rigion of the target gene.

The term "shRNA", as used herein, refers to an siRNA having a stem-loop structure, comprising a first and second regions complementary to one another, *i.e.*, sense and antisense strands. The degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The loop region of an shRNA is a single-stranded region intervening between the sense and antisense strands and can also be referred to as "intervening single-strand".

As use herein, the term "siD/R-NA" refers to a double-stranded polynucleotide molecule which is composed of both RNA and DNA, and includes hybrids and chimeras of RNA and DNA and prevents translation of a target mRNA. Herein, a hybrid indicates a molecule wherein a polynucleotide composed of DNA and a polynucleotied composed of RNA hybridize to each other to form the double-stranded molecule; whereas a chimera indicates that one or both of the strands composing the double stranded molecule can contain RNA and DNA. Standard techniques of introducing siD/R-NA into the cell are used. The siD/R-NA includes a sense nucleic acid sequence, an antisense nucleic acid sequence against an up-regulated marker gene, such as HRPC 1-36 or both. The siD/R-NA can be constructed such that a single transcript has both the sense and complementary antisense nucleic acid sequences from the target gene, *e.g.*, a hairpin. The siD/R-NA can either be a dsD/R-NA or shD/R-NA.

As used herein, the term "dsD/R-NA" refers to a construct of two molecules comprising complementary sequences to one another and that have annealed together via the complementary sequences to form a double-stranded polynucleotide molecule. The nucleotide sequence of two strands can comprise not only the "sense" or "antisense" polynucleotides sequence selected from a protein coding sequence of target gene sequence, but also polynucleotide having a nucleotide sequnence selected from non-coding region of the target gene. One or both of the two molecules constructing the dsD/R-NA are composed of both RNA and DNA (chimeric molecule), or alternatively, one of the molecules is composed of RNA and the other is composed of DNA (hybrid double-strand).

The term "shD/R-NA", as used herein, refers to an siD/R-NA having a stem-loop structure, comprising a first and second regions complementary to one another, *i.e.*, sense and antisense strands. The degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The loop region of an shD/R-NA is a single-stranded region intervening between the sense and antisense strands and can also be referred to as "intervening single-strand".

The term "polynucleotide" and "oligonucleotide" are used interchangeably herein unless otherwise specifically indicated and are referred to by their commonly accepted single-letter codes. The terms apply to nucleic acid (nucleotide) polymers in which one or more

nucleic acids are linked by ester bonding. The polynucleotide or oligonucleotide can be composed of DNA, RNA or a combination thereof.

As use herein, the term "double-stranded molecule" refers to a nucleic acid molecule that inhibits expression of a target gene including, for example, short interfering RNA (siRNA; *e.g.*, double-stranded ribonucleic acid (dsRNA) or small hairpin RNA (shRNA)) and short interfering DNA/RNA (siD/R-NA; *e.g.* double-stranded chimera of DNA and RNA (dsD/R-NA) or small hairpin chimera of DNA and RNA (shD/R-NA)).

An siRNA of an HRPC gene hybridizes to target mRNA and thereby decreases or inhibits production of the HRPC polypeptides encoded by the gene by associating with the normally single-stranded mRNA transcript, thereby interfering with translation and thus, expression of the protein. Thus, siRNA molecules of the invention can be defined by their ability to hybridize specifically to mRNA of a gene selected from HRPC 1-36 under stringent conditions. For the purposes of this invention the terms "hybridize" or "hybridize specifically" are used to refer the ability of two nucleic acid molecules to hybridize under "stringent hybridization conditions". The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid molecule will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but not detectably to other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, for example, at least 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 50°C.

In the context of the present invention, an siRNA is less than 500, 200, 100, 50, or 25 nucleotides in length. In some embodiments, an siRNA is 19-25 nucleotides in length. In order to enhance the inhibition activity of the siRNA, nucleotide "u" can be added to 3'end of the antisense strand of the target sequence. The number of "u"s to be added is at least 2, generally 2 to 10, for example, 2 to 5. The added "u"s form single strand at the 3'end of the antisense strand of the siRNA.

An siRNA of an HRPC gene can be directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. In these embodiments, the siRNA molecules of the invention are typically modified as described above for antisense molecules. Other modifications are also possible, for example, cholesterol-conjugated siRNAs have shown improved pharmacological properties (Song *et al. Nature Med.* 9:347-51 (2003)). Alternatively, a DNA encoding the siRNA can be carried in a vector.

Alternatively, a DNA encoding the siRNA can be carried in a vector (hereinafter, referred to as "si RNA vector"). Such vectors can be produced, for example, by cloning an HRPC gene target sequence into an expression vector having operatively-linked regulatory sequences (*e.g.* an RNA polymerase III transcription unit from the small nuclear RNA (snRNA) U6 or the human H1 RNA promoter) flanking the sequence in a manner that allows for expression (by transcription of the DNA molecule) of both strands (Lee, N.S., *et al.*, (2002) Nature Biotechnology 20 : 500-5.). An RNA molecule that is antisense to mRNA of an HRPC-associated gene is transcribed by a first promoter (*e.g.* a promoter sequence 3' of the cloned DNA) and an RNA molecule that is the sense strand for the mRNA of an HRPC-associated gene is transcribed by a second promoter (*e.g.* a promoter sequence 5' of the cloned DNA). The sense and antisense strands hybridize *in vivo* to generate siRNA constructs for silencing of the HRPC-associated gene. Alternatively, a construct can be utilized to create the sense and anti-sense strands of a single-stranded siRNA construct. In this case, a construct having secondary structure, *e.g.* hairpins, wherein a single transcript has both the sense and complementary antisense sequences from the target gene.

A loop sequence consisting of an arbitrary nucleotide sequence can be located between the sense and antisense sequence in order to form the hairpin loop structure. Thus, the present invention also provides siRNA having the general formula 5'-[A]-[B]-[A']-3', wherein [A] is a ribonucleotide sequence corresponding to a sequence that specifically hybridizes to an mRNA or a cDNA of gene selected from HRPC 1-36. In some embodiments,

[A] is a ribonucleotide sequence corresponding a gene selected from HRPC 1-36. [B] is a ribonucleotide sequence consisting of 3 to 23 nucleotides, and [A'] is a ribonucleotide sequence consisting of the complementary sequence of [A]. The region [A] hybridizes to [A'], and then a loop consisting of region [B] is formed. The loop sequence can be 3 to 23 nucleotide in length. The loop sequence, for example, can be selected from group consisting of sequences shown below (http://www.ambion.com/techlib/tb/tb_506.html). Furthermore, loop sequence consisting of 23 nucleotides also provides active siRNA (Jacque, J.M., *et al.* (2002) Nature 418: 435-8.).

- CCC, CCACC or CCACACC: Jacque, J.M.*et al.* (2002) Nature, Vol. 418: 435-8.

- UUCG: Lee, N.S., *et al.* (2002) Nature Biotechnology 20 : 500-5. Fruscoloni, P., *et al.* (2003) Proc. Natl. Acad. Sci. USA 100(4): 1639-44.

- UUCAAGAGA: Dykxhoorn, D. M., *et al.* (2002) Nature Reviews Molecular Cell Biology 4: 457-67.

Accordingly, the loop sequence can be selected from group consisting of, CCC, UUCG, CCACC, CCACACC, and UUCAAGAGA. An exemplified loop sequence is UUCAAGAGA ("ttcaagaga" in DNA).

Exemplary hairpin siRNA suitable for use in the context of the present invention include: (i) an ANLN-siRNA consisting of the sequence of 5'-ccaguugagucgacaucug-[B]-cagaugucgacucaacugg-3' (whose target sequence is shown in SEQ ID NO: 19); and (ii) SNRPE-siRNAs consisting of the sequence of 5'-ggaaagaaugaagugccuu-[B]-aaggcacuucauucuuucc-3' (whose target sequence is shown in SEQ ID NO: 17) or 5'-ggugaaugcagaaguguau-[B]-auacacuucugcauucacc-3' (whose target sequence is shown in SEQ ID NO: 18).

The nucleotide sequence of suitable siRNAs can be designed using an siRNA design computer program available from the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). The computer program selects nucleotide sequences for siRNA synthesis based on the following protocol.

Selection of siRNA Target Sites:

1. Beginning with the AUG start codon of the object transcript, scan downstream for AA dinucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19

nucleotides as potential siRNA target sites. Tuschl, *et al.* don't recommend against designing siRNA to the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within 75 bases) as these regions can be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex.

2. Compare the potential target sites to the human genome database and eliminate from consideration any target sequences with significant homology to other coding sequences. The homology search can be performed using BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/

3. Select qualifying target sequences for synthesis. At Ambion, several target sequences can be selected along the length of the gene to evaluate.

Standard techniques for introducing siRNA into the cell can be used. For example, an siRNA of HRPC associated genes (*e.g.* ANLN or SNRPE) can be directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. In these embodiments, the siRNA molecules of the present invention are typically modified as described above for antisense molecules. Other modifications are also possible, for example, cholesterol-conjugated siRNAs have shown improved pharmacological properties (Song *et al.*, Nature Med 2003, 9:347-51).

As used herein, the term "complementary" refers to Watson-Crick or Hoogsteen base pairing between nucleotides units of a nucleic acid molecule, and the term "binding" means the physical or chemical interaction between two nucleic acids or compounds or associated nucleic acids or compounds or combinations thereof. When the polynucleotide comprises modified nucleotides and/or non-phosphodiester linkages, these polynucleotides can also bind each other as same manner. Generally, complementary nucleic acid sequences hybridize under appropriate conditions to form stable duplexes containing few or no mismatches. For the purposes of this invention, two sequences having 5 or fewer mismatches are considered to be complementary. Furthermore, the sense strand and antisense strand of the isolated nucleotide of the present invention, can form double stranded nucleotide or hairpin loop structure by the hybridization.

The double-stranded molecules of the invention can contain one or more modified nucleotides and/or non-phosphodiester linkages. Chemical modifications well known in the

art are capable of increasing stability, availability, and/or cell uptake of the double-stranded molecule. The skilled person will be aware of other types of chemical modification which can be incorporated into the present molecules (WO03/070744; WO2005/045037). In one embodiment, modifications can be used to provide improved resistance to degradation or improved uptake. Examples of such modifications include phosphorothioate linkages, 2'-O-methyl ribonucleotides (especially on the sense strand of a double-stranded molecule), 2'-deoxy-fluoro ribonucleotides, 2'-deoxy ribonucleotides, "universal base" nucleotides, 5'-C-methyl nucleotides, and inverted deoxyabasic residue incorporation (US20060122137).

In another embodiment, modifications can be used to enhance the stability or to increase targeting efficiency of the double-stranded molecule. Modifications include chemical cross linking between the two complementary strands of a double-stranded molecule, chemical modification of a 3' or 5' terminus of a strand of a double-stranded molecule, sugar modifications, nucleobase modifications and/or backbone modifications, 2-fluoro modified ribonucleotides and 2'-deoxy ribonucleotides (WO2004/029212). In another embodiment, modifications can be used to increased or decreased affinity for the complementary nucleotides in the target mRNA and/or in the complementary double-stranded molecule strand (WO2005/044976). For example, an unmodified pyrimidine nucleotide can be substituted for a 2-thio, 5-alkynyl, 5-methyl, or 5-propynyl pyrimidine. Additionally, an unmodified purine can be substituted with a 7-deza, 7-alkyi, or 7-alkenyi purine. In another embodiment, when the double-stranded molecule is a double-stranded molecule with a 3' overhang, the 3'-terminal nucleotide overhanging nucleotides can be replaced by deoxyribonucleotides (Elbashir SM *et al.*, Genes Dev 2001 Jan 15, 15(2): 188-200). For further details, published documents such as US20060234970 are available. The present invention is not limited to these examples and any known chemical modifications can be employed for the double-stranded molecules of the present invention so long as the resulting molecule retains the ability to inhibit the expression of the target gene.

Furthermore, the double-stranded molecules of the invention can comprise both DNA and RNA, *e.g.*, dsD/R-NA or shD/R-NA. Specifically, a hybrid polynucleotide of a DNA strand and an RNA strand or a DNA-RNA chimera polynucleotide shows increased stability. Mixing of DNA and RNA, *i.e.*, a hybrid type double-stranded molecule consisting of a DNA strand (polynucleotide) and an RNA strand (polynucleotide), a chimera type double-stranded molecule comprising both DNA and RNA on any or both of the single strands (polynucleotides), or the like can be formed for enhancing stability of the double-

stranded molecule. The hybrid of a DNA strand and an RNA strand can be the hybrid in which either the sense strand is DNA and the antisense strand is RNA, or the opposite so long as it has an activity to inhibit expression of the target gene when introduced into a cell expressing the gene. The sense strand polynucleotide can be DNA and the antisense strand polynucleotide can be RNA. Also, the chimera type double-stranded molecule can be either where both of the sense and antisense strands are composed of DNA and RNA, or where any one of the sense and antisense strands is composed of DNA and RNA so long as it has an activity to inhibit expression of the target gene when introduced into a cell expressing the gene.

In order to enhance stability of the double-stranded molecule, the molecule can contain as much DNA as possible, whereas to induce inhibition of the target gene expression, the molecule is required to be RNA within a range to induce sufficient inhibition of the expression. As an example of the chimera type double-stranded molecule, an upstream partial region (*i.e.*, a region flanking to the target sequence or complementary sequence thereof within the sense or antisense strands) of the double-stranded molecule is RNA. The upstream partial region can indicate the 5' side (5'-end) of the sense strand and the 3' side (3'-end) of the antisense strand.

That is, in some embodiments, a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of sense strand and a region flanking to the 3'-end of antisense strand consists of RNA. For instance, the chimera or hybrid type double-stranded molecule of the present invention comprise following combinations.

sense strand:	5'-[DNA]-3'
	3'-(RNA)-[DNA]-5': antisense strand,
sense strand:	5'-(RNA)-[DNA]-3'
	3'-(RNA)-[DNA]-5': antisense strand, and
sense strand:	5'-(RNA)-[DNA]-3'
	3'-(RNA)-5': antisense strand.

The upstream partial region can be a domain consisting of 9 to 13 nucleotides counted from the terminus of the target sequence or complementary sequence thereto within the sense or antisense strands of the double-stranded molecules. Moreover, examples of such chimera type double-stranded molecules include those having a strand length of 19 to 21 nucleotides in which at least the upstream half region (5' side region for the sense strand and 3' side region for the antisense strand) of the polynucleotide is RNA and the other half is

DNA. In such a chimera type double-stranded molecule, the effect to inhibit expression of the target gene is much higher when the entire antisense strand is RNA (US20050004064).

In the present invention, the double-stranded molecule can form a hairpin, such as a short hairpin RNA (shRNA) and short hairpin consisting of DNA and RNA (shD/R-NA). The shRNA or shD/R-NA is a sequence of RNA or mixture of RNA and DNA making a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNA or shD/R-NA comprises the sense target sequence and the antisense target sequence on a single strand wherein the sequences are separated by a loop sequence. Generally, the hairpin structure is cleaved by the cellular machinery into dsRNA or dsD/R-NA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which match the target sequence of the dsRNA or dsD/R-NA.

Alternatively, a DNA encoding the siRNA can be carried in a vector (hereinafter, also referred to as "siRNA vector"). Such vectors can be produced, for example, by cloning a target HRPC associated gene sequence into an expression vector having operatively-linked regulatory sequences (*e.g.* a RNA polymerase III transcription unit from the small nuclear RNA (snRNA) U6 or the human H1 RNA promoter) flanking the sequence in a manner that allows for expression (by transcription of the DNA molecule) of both strands (Lee NS *et al.*, Nature Biotechnology 2002, 20: 500-5). For example, an RNA molecule that is antisense to mRNA of the HRPC associated gene is transcribed by a first promoter (*e.g.* a promoter sequence 3' of the cloned DNA) and an RNA molecule that is the sense strand for the mRNA of the HRPC associated gene is transcribed by a second promoter (*e.g.* a promoter sequence 5' of the cloned DNA). The sense and antisense strands hybridize *in vivo* to generate siRNA constructs for silencing the expression of the HRPC associated gene. Alternatively, a construct can be utilized to create the sense and anti-sense strands of a single-stranded siRNA construct. In this case, a construct having secondary structure, *e.g.* hairpin, is produced as a single transcript that comprises both the sense and complementary antisense sequences of the target gene.

Thus, the present pharmaceutical composition for treating or preventing HRPC comprises either the siRNA or a vector expressing the siRNA *in vivo*.

For introducing the siRNA vector into the cell, transfection-enhancing agent can be used. FuGENE6 (Roche diagnostics), Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen), and Nucleofector (Wako pure Chemical) are useful as the transfection-enhancing

agent. Therefore, the present pharmaceutical composition can further include such transfection-enhancing agents.

Alternatively, according to the present invention, use of small interfering RNA against a polynucleotide select from the group consisting of HRPC 1-36 for manufacturing a pharmaceutical composition for treating or preventing hormone-refractory prostate cancer is provided. Further, the present invention also provides small interfering RNA against a polynucleotide select from the group consisting of HRPC 1-36 for treating or preventing hormone-refractory prostate cancer.

The antisense oligonucleotide or siRNA of the present invention inhibits the expression of a polypeptide of the present invention, and is thereby useful for suppressing the biological activity of a polypeptide of the invention. Also, expression-inhibitors, comprising the antisense oligonucleotide or siRNA of the invention, are useful in the point that they can inhibit the biological activity of the polypeptide of the invention. Therefore, a composition comprising an antisense oligonucleotide or siRNA of the present invention is useful for treating or preventing an HRPC.

By HRPC associated genes target sequence is meant a nucleotide sequence that is identical to a portion of the HRPC associated genes (*i.e*, a polynucleotide within HRPC associated genes that are equal in length to and complementary to an siRNA). The target sequence can include the 5' untranslated (UT) region, the open reading frame (ORF) or the 3' untranslated region of the human HRPC associated genes.

Ribozymes:

Furthermore, the present invention provides ribozymes that reduce the expression of an HRPC gene selected from the group consisting of HRPC 1-36.

Generally, ribozymes are classified into large ribozymes and small ribozymes. A large ribozyme is known as an enzyme that cleaves the phosphate ester bond of nucleic acids. After the reaction with the large ribozyme, the reacted site consists of a 5'-phosphate and 3'-hydroxyl group. The large ribozyme is further classified into (1) group I intron RNA catalyzing transesterification at the 5'-splice site by guanosine; (2) group II intron RNA catalyzing self-splicing through a two step reaction via lariat structure; and (3) RNA component of the ribonuclease P that cleaves the tRNA precursor at the 5' site through hydrolysis. On the other hand, small ribozymes have a smaller size (about 40 bp) compared

to the large ribozymes and cleave RNAs to generate a 5'-hydroxyl group and a 2'-3' cyclic phosphate. Hammerhead type ribozymes (Koizumi *et al.*, FEBS Lett 228: 228 (1988)) and hairpin type ribozymes (Buzayan, Nature 323: 349-53 (1986); Kikuchi and Sasaki, Nucleic Acids Res 19: 6751-5 (1991)) are included in the small ribozymes. Methods for designing and constructing ribozymes are known in the art (see Koizumi *et al.*, FEBS Lett 228: 228 (1988); Koizumi *et al.*, Nucleic Acids Res. 17: 7059-71 (1989); Kikuchi and Sasaki, Nucleic Acids Res 19: 6751-5 (1991)). Thus, ribozymes inhibiting the expression of the polypeptides of any one of HRPC 1-36 of the present invention can also be constructed based on their sequence information and these conventional methods.

Ribozymes against an HRPC-associated up-regulated gene inhibit the expression of over-expressed HRPC-associated up-regulated protein and is thus useful for suppressing the biological activity of the protein. Therefore, the ribozymes are useful in treating or preventing HRPC.

In the present invention, the inhibitory nucleic acids can be administered to the subject either as a naked nucleic acids, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector which expresses the inhibitory nucleic acids.

Suitable delivery reagents for administration in conjunction with the present inhibitory nucleic acids include the Mirus Transit TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; or polycations (e.g., polylysine), or liposomes. A preferred delivery reagent is a liposome.

Liposomes can aid in the delivery of the inhibitory nucleic acids to a particular tissue, such as retinal or tumor tissue, and can also increase the blood half-life of the inhibitory nucleic acids. Liposomes suitable for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the liposomes in the blood stream. A variety of methods are known for preparing liposomes, for example as described in Szoka et al., Ann Rev Biophys Bioeng 1980, 9: 467; and US Pat. Nos. 4,235,871; 4,501,728; 4,837,028; and 5,019,369, the entire disclosures of which are herein incorporated by reference.

Preferably, the liposomes encapsulating the present inhibitory nucleic acids comprise a ligand molecule that can deliver the liposome to the cancer site. Ligands which bind to

receptors prevalent in tumor cells, such as monoclonal antibodies that bind to tumor antigens, are preferred.

Particularly preferably, the liposomes encapsulating the present inhibitory nucleic acids are modified so as to avoid clearance by the mononuclear macrophage and reticuloendothelial systems, for example, by having opsonization-inhibition moieties bound to the surface of the structure. In one embodiment, a liposome of the invention can comprise both opsonization-inhibition moieties and a ligand.

Opsonization-inhibiting moieties for use in preparing the liposomes of the invention are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, e.g., by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer which significantly decreases the uptake of the liposomes by the macrophage-monocyte system ("MMS") and reticuloendothelial system ("RES"); e.g., as described in US Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference. Liposomes modified with opsonization-inhibition moieties thus remain in the circulation much longer than unmodified liposomes. For this reason, such liposomes are sometimes called "stealth" liposomes.

Stealth liposomes are known to accumulate in tissues fed by porous or "leaky" microvasculature. Thus, target tissue characterized by such microvasculature defects, for example, solid tumors, will efficiently accumulate these liposomes; see Gabizon et al., Proc Natl Acad Sci USA 1988, 18: 6949-53. In addition, the reduced uptake by the RES lowers the toxicity of stealth liposomes by preventing significant accumulation in liver and spleen. Thus, liposomes of the invention that are modified with opsonization-inhibition moieties can deliver the present inhibitory nucleic acids to tumor cells.

Opsonization inhibiting moieties suitable for modifying liposomes are preferably water-soluble polymers with a molecular weight from about 500 to about 40,000 daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; e.g., methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids;

polyalcohols, e.g., polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM.sub.1. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, e.g., reacted with derivatives of carbonic acids with resultant linking of carboxylic groups.

Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated liposomes".

The opsonization inhibiting moiety can be bound to the liposome membrane by any one of numerous well-known techniques. For example, an N-hydroxysuccinimide ester of PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be derivatized with a stearylamine lipid-soluble anchor via reductive amination using Na(CN)BH. sub. 3 and a solvent mixture such as tetrahydrofuran and water in a 30:12 ratio at 60. degree. C.

Vectors expressing inhibitory nucleic acids of the invention are discussed above. Such vectors expressing at least one inhibitory nucleic acids of the invention can also be administered directly or in conjunction with a suitable delivery reagent, including the Mirus Transit LT1 lipophilic reagent; lipofectin; lipofectamine; cellfectin; polycations (e.g., polylysine) or liposomes. Methods for delivering recombinant viral vectors, which express inhibitory nucleic acids of the invention, to an area of cancer in a patient are within the skill of the art.

The inhibitory nucleic acids of the invention can be administered to the subject by any means suitable for delivering the inhibitory nucleic acids into cancer sites. For example, the inhibitory nucleic acids can be administered by gene gun, electroporation, or by other suitable parenteral or enteral administration routes.

Suitable enteral administration routes include oral, rectal, or intranasal delivery.

Suitable parenteral administration routes include intravascular administration (e.g., intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue injection (e.g., peri-tumoral and intra-tumoral injection, intra-retinal injection, or subretinal injection); subcutaneous injection or deposition including subcutaneous infusion (such as by osmotic pumps); direct application to the area at or near the site of cancer, for example by a catheter or other placement device (e.g., a retinal pellet or a suppository or an implant comprising a porous, non-porous, or gelatinous material); and inhalation. It is preferred that injections or infusions of the inhibitory nucleic acids or vector be given at or near the site of cancer.

The inhibitory nucleic acids of the invention can be administered in a single dose or in multiple doses. Where the administration of the inhibitory nucleic acids of the invention is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. Injection of the agent directly into the tissue is at or near the site of cancer preferred. Multiple injections of the agent into the tissue at or near the site of cancer are particularly preferred.

One skilled in the art can also readily determine an appropriate dosage regimen for administering the inhibitory nucleic acids of the invention to a given subject. For example, the inhibitory nucleic acids can be administered to the subject once, for example, as a single injection or deposition at or near the cancer site. Alternatively, the inhibitory nucleic acids can be administered once or twice daily to a subject for a period of from about three to about twenty-eight days, more preferably from about seven to about ten days. In a preferred dosage regimen, the inhibitory nucleic acids are injected at or near the site of cancer once a day for seven days. Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of an inhibitory nucleic acids administered to the subject can comprise the total amount of an inhibitory nucleic acids administered over the entire dosage regimen.

Antibodies:

Function of one or more gene products of the genes over-expressed in HRPC can also be inhibited by administering a compound that binds to or otherwise inhibits the function of the gene products. For example, the compound is an antibody which binds to the over-expressed gene product or gene products.

The present invention refers to the use of antibodies, particularly antibodies against a

protein encoded by an up-regulated HRPC marker gene, or a fragment of such an antibody. As used herein, the term "antibody" refers to an immunoglobulin molecule having a specific structure, that interacts (*i.e.* binds) only with the antigen that was used for synthesizing the antibody (*i.e.* the gene product of an up-regulated marker) or with an antigen closely related thereto. Furthermore, an antibody can be a fragment of an antibody or a modified antibody, so long as it binds to one or more of the proteins encoded by the marker genes. For instance, the antibody fragment can be Fab, $F(ab')_2$, Fv, or single chain Fv (scFv), in which Fv fragments from H and L chains are ligated by an appropriate linker (Huston J. S. *et al.* Proc. Natl. Acad. Sci. U.S.A. 85:5879-83 (1988)). More specifically, an antibody fragment can be generated by treating an antibody with an enzyme, such as papain or pepsin. Alternatively, a gene encoding the antibody fragment can be constructed, inserted into an expression vector, and expressed in an appropriate host cell (*see*, for example, Co M. S. *et al.* J. Immunol. 152:2968-76 (1994); Better M. and Horwitz A. H. Methods Enzymol. 178:476-96 (1989); Pluckthun A. and Skerra A. Methods Enzymol. 178:497-515 (1989); Lamoyi E. Methods Enzymol. 121:652-63 (1986); Rousseaux J. *et al.* Methods Enzymol. 121:663-9 (1986); Bird R. E. and Walker B. W. Trends Biotechnol. 9:132-7 (1991)).

An antibody can be modified by conjugation with a variety of molecules, such as polyethylene glycol (PEG). The present invention provides such modified antibodies. The modified antibody can be obtained by chemically modifying an antibody. Such modification methods are conventional in the field.

Alternatively, an antibody can comprise as a chimeric antibody having a variable region derived from a nonhuman antibody and a constant region derived from a human antibody, or a humanized antibody, comprising a complementarity determining region (CDR) derived from a nonhuman antibody, the frame work region (FR) derived from a human antibody and the constant region. Such antibodies can be prepared by using known technologies. Humanization can be performed by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody (*see*, *e.g.* Verhoeyen *et al.*, *Science* 239:1534-6 (1988)). Accordingly, such humanized antibodies are chimeric antibodies, wherein an intact human variable domain has been substituted by the corresponding sequence from a non-human species.

Fully human antibodies comprising human variable regions in addition to human framework and constant regions can also be used. Such antibodies can be produced using

various techniques known in the art. For example *in vitro* methods involve use of recombinant libraries of human antibody fragments displayed on bacteriophage (*e.g.* Hoogenboom & Winter, J. Mol. Biol. 227:381-8 (1991)). Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, *e.g.* mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. This approach is described, *e.g.* in U.S. Patent Nos. 6,150,584, 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016.

Alternatively, according to the present invention, use of an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of HRPC 1-36 for manufacturing a pharmaceutical composition for treating or preventing hormone-refractory prostate cancer is provided. Further, the present invention also provides an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of HRPC 1-36 for treating or preventing hormone-refractory prostate cancer.

Cancer therapies directed at specific molecular alterations that occur in cancer cells have been validated through clinical development and regulatory approval of anti-cancer drugs such as trastuzumab (Herceptin) for the treatment of advanced breast cancer, imatinib methylate (Gleevec) for chronic myeloid leukemia, gefitinib (Iressa) for non-small cell lung cancer (NSCLC), and rituximab (anti-CD20 mAb) for B-cell lymphoma and mantle cell lymphoma (Ciardiello F & Tortora G. Clin Cancer Res. 2001 Oct;7(10):2958-70. Review.; Slamon DJ, *et al.* N Engl J Med. 2001 Mar 15;344(11):783-92.; Rehwald U, *et al.* Blood. 2003 Jan 15;101(2):420-4.; Fang G, *et al.* (2000). Blood, 96, 2246-53.). These drugs are clinically effective and better tolerated than traditional anti-cancer agents because they target only transformed cells. Hence, such drugs not only improve survival and quality of life for cancer patients, but also validate the concept of molecularly targeted cancer therapy. Furthermore, targeted drugs can enhance the efficacy of standard chemotherapy when used in combination with it (Gianni L. (2002). Oncology, 63 Suppl 1, 47-56.; Klejman A, *et al.* (2002). Oncogene, 21, 5868-76.). Therefore, future cancer treatments will probably involve combining conventional drugs with target-specific agents aimed at different characteristics of tumor cells such as angiogenesis and invasiveness.

These modulatory methods can be performed *ex vivo* or *in vitro* (*e.g.* by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.* by administering the agent to a subject).

The methods involve administering a protein or combination of proteins or a nucleic acid molecule or combination of nucleic acid, molecules as therapy to counteract aberrant expression of the differentially expressed genes or aberrant activity of their gene products.

Diseases and disorders that are characterized by increased (relative to a subject not suffering from the disease or disorder) expression levels or biological activity in HRPC 1-36 genes and gene products, respectively, can be treated with therapeutics that antagonize (*i.e.* reduce or inhibit) activity of the over-expressed gene or genes. Therapeutics that antagonize activity can be administered therapeutically or prophylactically.

Accordingly, therapeutics that can be utilized in the context of the present invention including, *e.g.* (*i*) a polypeptide encoded by the over-expressed or under-expressed gene or genes, or analogs, derivatives, fragments or homologs thereof; (*ii*) antibodies to the overexpressed gene products; (*iii*) nucleic acids encoding the under-expressed gene products; (*iv*) antisense nucleic acids or nucleic acids that are "dysfunctional" (*i.e.* due to a heterologous insertion within the nucleic acids of one or more over-expressed gene or genes); (*v*) small interfering RNA (siRNA); or (*vi*) modulators (*i.e.* inhibitors, agonists and antagonists that alter the interaction between an over/under-expressed polypeptide and its binding partner). The dysfunctional antisense molecules can be utilized to knockout the endogenous function of a polypeptide by homologous recombination (*see*, *e.g.* Capecchi, *Science* 244: 1288-92 1989).

Diseases and disorders that are characterized by decreased expression levels or biological activity in HRPC gene and gene products can be treated with therapeutics that increase (*i.e.* are agonists to) activity. Therapeutics that up-regulate activity can be administered in a therapeutic or prophylactic manner. Therapeutics that can be utilized include, but are not limited to, a polypeptide (or analogs, derivatives, fragments or homologs thereof) or an agonist that increases bioavailability.

Increased or decreased levels of HRPC-associated nucleic acids or proteins can be readily detected by quantifying peptide and/or RNA, by obtaining a patient tissue sample (*e.g.* from biopsy tissue) and assaying it *in vitro* for RNA or peptide levels, structure and/or activity of the expressed peptides (or mRNAs of a gene whose expression is altered). Methods that are well-known within the art include, but are not limited to, immunoassays (*e.g.* by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide

gel electrophoresis, immunocytochemistry, *etc.*) and/or hybridization assays to detect expression of mRNAs (*e.g.* Northern assays, dot blots, *in situ* hybridization, *etc.*).

Prophylactic administration occurs prior to the manifestation of clinical symptoms of disease, such that a disease or disorder is prevented or, alternatively, delayed in its progression.

Therapeutic methods of the present invention can include the step of contacting a cell with an agent that modulates one or more of the activities of the gene products of the differentially expressed genes. Examples of agents that modulate protein activity include, but are not limited to, nucleic acids, proteins, naturally-occurring cognate ligands of such proteins, peptides, peptidomimetics, and other small molecules. For example, a suitable agent can stimulate one or more protein activities of one or more differentially under-expressed genes.

Vaccinating against prostate cancer:

The present invention also relates to a method of treating or preventing HRPC in a subject comprising the step of administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid selected from the group consisting of HRPC 1-36 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding such a polypeptide or fragment thereof. Administration of the polypeptide induces an anti-tumor immunity in a subject. To induce anti-tumor immunity, a polypeptide encoded by a nucleic acid selected from the group consisting of HRPC 1-36 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding such a polypeptide or fragment thereof is administered (*e.g.*, intradermally, subcutaneously) to subject in need thereof. The polypeptide or the immunologically active fragments thereof are useful as vaccines against HRPC. In some cases, the proteins or fragments thereof can be administered in a form bound to the T cell receptor (TCR) or presented by an antigen presenting cell (APC), such as macrophage, dendritic cell (DC), or B-cells. Due to the strong antigen presenting ability of DC, the use of DC is most preferable among the APCs.

In the present invention, a vaccine against HRPC refers to a substance that has the ability to induce anti-tumor immunity upon inoculation into animals. According to the present invention, polypeptides encoded by a nucleic acid selected from the group consisting of HRPC 1-36 or fragments thereof were suggested to be HLA-A24 or HLA-A*0201 restricted epitope peptides that induce potent and specific immune response against HRPC

cells expressing HRPC 1-36. Thus, the present invention also encompasses method of inducing anti-tumor immunity using the polypeptides. In general, anti-tumor immunity includes immune responses such as follows:

- induction of cytotoxic lymphocytes against tumors,
- induction of antibodies that recognize tumors, and
- induction of anti-tumor cytokine production.

Therefore, when a certain protein induces any one of these immune responses upon inoculation into an animal, the protein is determined to have anti-tumor immunity inducing effect. The induction of the anti-tumor immunity by a protein can be detected by observing *in vivo* or *in vitro* the response of the immune system in the host against the protein.

For example, a method for detecting the induction of cytotoxic T lymphocytes is well known. Specifically a foreign substance that enters the living body is presented to T cells and B cells by the action of antigen presenting cells (APCs). T cells that respond to the antigen presented by the APCs in an antigen specific manner differentiate into cytotoxic T cells (or cytotoxic T lymphocytes; CTLs) due to stimulation by the antigen, and then proliferate (this is referred to as activation of T cells). Therefore, CTL induction by a certain peptide can be evaluated by presenting the peptide to a T cell via an APC, and detecting the induction of CTLs. Furthermore, APCs have the effect of activating CD4+ T cells, CD8+ T cells, macrophages, eosinophils, and NK cells. Since CD4+ T cells and CD8+ T cells are also important in anti-tumor immunity, the anti-tumor immunity inducing action of the peptide can be evaluated using the activation effect of these cells as indicators.

A method for evaluating the inducing action of CTLs using dendritic cells (DCs) as the APC is well known in the art. DCs are a representative APCs having the strongest CTL-inducing action among APCs. In this method, the test polypeptide is initially contacted with DCs, and then the DCs are contacted with T cells. Detection of T cells having cytotoxic effects against the cells of interest after the contact with DC shows that the test polypeptide has an activity of inducing the cytotoxic T cells. Activity of CTLs against tumors can be detected, for example, using the lysis of ^{51}Cr-labeled tumor cells as the indicator. Alternatively, the method of evaluating the degree of tumor cell damage using ^{3}H-thymidine uptake activity or LDH (lactate dehydrogenase)-release as the indicator is also well known.

Apart from DCs, peripheral blood mononuclear cells (PBMCs) can also be used as

the APC. The induction of CTLs has been reported to be enhanced by culturing PBMCs in the presence of GM-CSF and IL-4. Similarly, CTLs have been shown to be induced by culturing PBMCs in the presence of keyhole limpet hemocyanin (KLH) and IL-7.

Test polypeptides confirmed to possess CTL -inducing activity by these methods are deemed to be polypeptides having DC activation effect and subsequent CTL -inducing activity. Therefore, polypeptides that induce CTLs against tumor cells are useful as vaccines against tumors. Furthermore, APCs that have acquired the ability to induce CTLs against tumors through contact with the polypeptides are also useful as vaccines against tumors. Furthermore, CTLs, that have acquired cytotoxicity due to presentation of the polypeptide antigens by APCs can also be used as vaccines against tumors. Such therapeutic methods for tumors using anti-tumor immunity due to APCs and CTLs are referred to as cellular immunotherapy.

Generally, when using a polypeptide for cellular immunotherapy, efficiency of the CTL-induction is known to be increased by combining a plurality of polypeptides having different structures and contacting them with DCs. Therefore, when stimulating DCs with protein fragments, it is advantageous to use a mixture of multiple types of fragments.

Alternatively, the induction of anti-tumor immunity by a polypeptide can be confirmed by observing the induction of antibody production against tumors. For example, when antibodies against a polypeptide are induced in a laboratory animal immunized with the polypeptide, and when growth of tumor cells is suppressed by those antibodies, the polypeptide is deemed to have the ability to induce anti-tumor immunity.

Anti-tumor immunity is induced by administering the vaccine of this invention, and the induction of anti-tumor immunity enables treatment and prevention of HRPC. Therapy against cancer or prevention of the onset of cancer includes any of the following steps, such as inhibition of the growth of cancerous cells, involution of cancer, and suppression of occurrence of cancer. A decreases in mortality and mortality of individuals having cancer, decrease in the levels of tumor markers in the blood, alleviation of detectable symptoms accompanying cancer, and such are also included in the therapy or prevention of cancer. Such therapeutic and preventive effects can be statistically significant. For example, in observation, the therapeutic and preventive effects are a significance level of 5% or less, wherein the therapeutic or preventive effect of a vaccine against cell proliferative diseases is compared to

a control without vaccine administration. For example, Student's t-test, the Mann-Whitney U-test, or ANOVA can be used for statistical analysis.

The above-mentioned protein having immunological activity or a vector encoding the protein can be combined with an adjuvant. An adjuvant refers to a compound that enhances the immune response against the protein when administered together (or successively) with the protein having immunological activity. Exemplary adjuvants include, but are not limited to, cholera toxin, salmonella toxin, alum, and such. Furthermore, the vaccine of this invention can be combined appropriately with a pharmaceutically acceptable carrier. Examples of such carriers include sterilized water, physiological saline, phosphate buffer, culture fluid, and such. Furthermore, the vaccine can contain as necessary, stabilizers, suspensions, preservatives, surfactants, and such. The vaccine can be administered systemically or locally. Vaccine administration can be performed by single administration, or boosted by multiple administrations.

When using an APC or CTL as the vaccine of this invention, tumors can be treated or prevented, for example, by the *ex vivo* method. More specifically, PBMCs of the subject receiving treatment or prevention are collected, the cells are contacted with the polypeptide *ex vivo*, and following the induction of APCs or CTLs, the cells can be administered to the subject. APCs can be also induced by introducing a vector encoding the polypeptide into PBMCs *ex vivo*. APCs or CTLs induced *in vitro* can be cloned prior to administration. By cloning and growing cells having high activity of damaging target cells, cellular immunotherapy can be performed more effectively. Furthermore, APCs and CTLs isolated in this manner can be used for cellular immunotherapy not only against individuals from whom the cells are derived, but also against similar types of tumors from other individuals.

Furthermore, a pharmaceutical composition for treating or preventing a cell proliferative disease, such as cancer, comprising a pharmaceutically effective amount of the polypeptide of the present invention is provided. The pharmaceutical composition can be used for raising anti tumor immunity.

Pharmaceutical compositions for inhibiting HRPC:

In the context of the present invention, suitable pharmaceutical formulations include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration, or for

administration by inhalation or insufflation. Administration can be intravenous. The formulations are optionally packaged in discrete dosage units.

Pharmaceutical formulations suitable for oral administration include capsules, cachets or tablets, each containing a predetermined amount of active ingredient. Suitable formulations also include powders, granules, solutions, suspensions and emulsions. The active ingredient is optionally administered as a bolus electuary or paste. Tablets and capsules for oral administration can contain conventional excipients, such as binding agents, fillers, lubricants, disintegrant and/or wetting agents. A tablet can be made by compression or molding, optionally with one or more formulational ingredients. Compressed tablets can be prepared by compressing in a suitable machine the active ingredients in a free-flowing form, such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active and/or dispersing agent. Molded tablets can be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets can be coated according to methods well known in the art. Oral fluid preparations can be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which can include edible oils), and/or preservatives. The tablets can optionally be formulated so as to provide slow or controlled release of the active ingredient therein. A package of tablets can contain one tablet to be taken on each day of the month.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions, optionally contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; as well as aqueous and non-aqueous sterile suspensions including suspending agents and/or thickening agents. The formulations can be presented in unit dose or multi-dose containers, for example as sealed ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Alternatively, the formulations can be presented for continuous infusion. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations suitable for rectal administration include suppositories with standard carriers such as cocoa butter or polyethylene glycol. Formulations suitable for topical administration in the mouth, for example buccally or sublingually, include lozenges, containing the active ingredient in a flavored base such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a base such as gelatin and glycerin or sucrose and acacia. For intra-nasal administration the compounds of the invention can be used as a liquid spray, a dispersible powder or in the form of drops. Drops can be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents and/or suspending agents.

For administration by inhalation the compounds can be conveniently delivered from an insufflator, nebulizer, pressurized packs or other convenient means of delivering an aerosol spray. Pressurized packs can comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichiorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit can be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the compounds can take the form of a dry powder composition, for example a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition can be presented in unit dosage form, for example, as capsules, cartridges, gelatin or blister packs from which the powder can be administered with the aid of an inhalator or insufflators.

Other formulations include implantable devices and adhesive patches; which release a therapeutic agent.

When desired, the above described formulations, adapted to give sustained release of the active ingredient, can be employed. The pharmaceutical compositions can also contain other active ingredients such as antimicrobial agents, immunosuppressants and/or preservatives.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention can include other agents conventional in the art with regard to the type of formulation in question. For example, formulations suitable for oral administration can include flavoring agents.

Unit dosage formulations can contain an effective dose, as recited below, or an appropriate fraction thereof, of the active ingredient.

For each of the aforementioned conditions, the compositions, *e.g.* polypeptides and organic compounds, can be administered orally or via injection at a dose ranging from about 0.1 to about 250 mg/kg per day. The dose range for adult humans is generally from about 5 mg to about 17.5 g/day, for example, about 5 mg to about 10 g/day, for example, about 100 mg to about 3 g/day. Tablets or other unit dosage forms of presentation provided in discrete units can conveniently contain an amount which is effective at such dosage or as a multiple of the same, for instance, units containing about 5 mg to about 500 mg, usually from about 100 mg to about 500 mg.

The dose employed will depend upon a number of factors, including the age and sex of the subject, the precise disorder being treated, and its severity. Also the route of administration can vary depending upon the condition and its severity. In any event, appropriate and optimum dosages can be routinely calculated by those skilled in the art, taking into consideration the above-mentioned factors.

Aspects of the present invention are described in the following examples, which are not intended to limit the scope of the invention described in the claims. The following examples illustrate the identification and characterization of genes differentially expressed in HRPC or HSPC cells.

EXAMPLE

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: General Methods

Patients and tissue samples

Tissue samples were obtained with informed consent from 43 HRPC patients undergoing prostatic needle biopsy, bone biopsy, TUR-P (transurethral resection of the prostate), and autopsy. Clinical HRPC was defined by elevation of serum PSA levels at three consecutive times and/or enlargement of tumor in spite of androgen-ablation therapy. All of the samples were embedded in TissueTek OCT medium (Sakura, Tokyo, Japan) immediately after tissue procurement and stored at -80°C until their use. Histopathological diagnoses were

made by a single pathologist (M.F.) before LMM, and HE-stained sections from adjacent frozen tissues were prepared to confirm the histological diagnosis. Among the 43 HRPC patients the present inventors obtained, 25 primary and metastatic tumor specimens from 18 HRPC patients had sufficient amounts and good quality of RNAs for our microarray analysis. Simultaneously, 10 hormone-sensitive or naïve prostate cancers (HSPCs) were also microdissected from 10 untreated operable cases undergoing radical prostatectomy, and normal prostatic epithelial cells were also microdissected from one benign prostatic hyperplasia (BPH) patient and four bladder cancer patients, where the inventors confirmed no apparent prostate cancers or PINs (prostatic intraepithelial neoplasia) histopathologically.

Laser microbeam microdissection and T7-based RNA amplification

LMM and T7-based RNA amplification were performed as described previously (Ashida S, Cancer Res. 2004 Sep 1;64(17):5963-72.). 2.5μg each of amplified RNA were labeled by reverse transcription with Cy5-dCTP for cancer cells or Cy3-dCTP for normal cells (Amersham Biosciences, Buckinghamshire, UK), as described previously (Ashida S, Cancer Res. 2004 Sep 1;64(17):5963-72.).

cDNA microarray analysis and acquisition of data

The present inventors fabricated a genome-wide cDNA microarray with 36,864 cDNAs selected from the UniGene database (build no.131) of the National Center for Biotechnology Information (NCBI). Construction, hybridization, washing, and scanning were carried out according to methods described previously (Ashida S, Cancer Res. 2004 Sep 1;64(17):5963-72.). Signal intensities of Cy3 and Cy5 from the 36,864 spots were quantified and analyzed by substituting backgrounds with ArrayVision software (Imaging Research, Inc., St. Catharines, Ontario, Canada). Subsequently, the fluorescent intensities of Cy5 (cancer) and Cy3 (normal control) for each target spot were adjusted so that the mean Cy3/Cy5 ratio of 52 housekeeping genes was equal to one. Since data with low-signal intensities are less reliable, the inventors determined a cut-off value on each slide, and they excluded genes from further analysis when both the Cy3 and the Cy5 dyes yielded signal intensities lower than that of the cut-off value. For other genes, the present inventors calculated the Cy5/Cy3 ratio using the raw data of each sample.

Clustering and statistical analysis for genome-wide gene expression profiles

The present inventors applied a hierarchical clustering method to both genes and tumors, excluding genes, for which both Cy3- and Cy5-fluorescence intensities were below

the cut-off value. To obtain reproducible clusters for classification of the 35 tumors, this inventors selected 254 genes for which valid data were obtained in 80% of the experiments and whose expression ratios varied by SDs (standard deviations) of more than 1.75. The analysis was performed with web-available software (Cluster and TreeView) written by Eisen (Sherlock G, *et al.*, Nucleic Acids Res. 2001 Jan 1;29(1):152-5., Gollub J, *et al.*, Nucleic Acids Res. 2003 Jan 1;31(1):94-6., Ball CA, *et al.*, Nucleic Acids Res. 2005 Jan 1;33 (Database issue): D580-2.; http://genome-www5.stanford.edu/MicroArray/SMD/restech.html). Before applying the clustering algorithm, the present inventors log-transformed the fluorescence ratio for each spot and then median-centered the data for each sample to remove experimental biases. Then the inventors applied a random permutation test to identify genes that were expressed at a significantly different level between the two groups (HRPC vs HSPC). The present inventors carried out permutation tests to estimate the ability of individual genes to distinguish these two groups: samples were randomly permutated 10,000 times. The present inventors selected 106 genes for which valid data were obtained by P-value of less than 0.0001, two-group gap of more than 1.5, and one group present of more than 60%.

Semi-quantitative RT-PCR

Total RNA was extracted using RNeasy Kit (QIAGEN, Valencia, CA) according to manufacture's instruction, treated with DNase I (Roche Diagnostic, Mannheim, Germany), and reversely transcribed to single-stranded cDNA using random hexamer or oligo d(T)12-18 primer with Superscript reverse transcriptase II (Invitrogen, Carlsbad, CA). The present inventors prepared appropriate dilutions of each single-strand cDNA followed by normalizing cDNA content using β-actin (ACTB) as a quantitative control, demonstrating PCR reaction using single strand cDNA as PCR templates. The primers of each transcripts are shown below:

ACTB (forward 5'-TTGGCTTGACTCAGGATTTA-3' (SEQ ID NO: 5),
reverse 5'-ATGCTATCACCTCCCCTGTG-3' (SEQ ID NO: 6)),

SNRPE (forward 5'-CAAGTGAATATGCGGATAGAAGG-3' (SEQ ID NO: 7),
reverse 5'-CCATCTTGTAGTAACACGAGGGT-3' (SEQ ID NO: 8)),

ANLN (forward 5'-GCTGCGTAGCTTACAGACTTAGC-3' (SEQ ID NO: 9),
reverse: 5'-AAGGCGTTTAAAGGTGATAGGTG-3' (SEQ ID NO: 10)),

AR (forward: 5'-GTGCTGTCCTTGGAATTAATCTG-3' (SEQ ID NO: 11),

reverse: 5'-AACAGAACACTAGCGCTTGGAG-3' (SEQ ID NO: 12)),
PSA (forward: 5'-GATAGGATGGGGTGTCTGTGTT-3' (SEQ ID NO: 13),
reverse: 5'-CAGTCCCTCTCCTTACTTCATCC-3' (SEQ ID NO: 14)),
NKX3.1 (forward: 5'-TGCAAGGATTACACATTTCACTG-3' (SEQ ID NO: 15),
reverse: 5'-AGCAGGGTTTGTTATGCATGTAG-3' (SEQ ID NO: 16)),
TMEM46 (forward: 5'-GGCTTATTCTTCAGGCACTAAGG-3' (SEQ ID NO: 30),
reverse: 5'-AGCAGTTGGAAATGTACTTGCAC-3' (SEQ ID NO:31)),
CDKN2C (forward: 5'-GCAGCTAAATTTTCTGAAACTGC-3' (SEQ ID NO: 32),
reverse: 5'-AGACAAAAGCATCAGGACAAACC-3' (SEQ ID NO: 33)),
DTL (forward: 5'-TGACCAATATCTGCCAGTAACG-3' (SEQ ID NO: 34),
reverse: 5'-CAGGATCAGCTCAAAGTCTGACA-3' (SEQ ID NO: 35)),
PTTG2 (forward: 5'-CTGCCTCAGATGACGCCTAT-3' (SEQ ID NO: 36),
reverse: 5'-ACATCCAGGGTCGACAGAATG-3' (SEQ ID NO: 37)),
SMC4 (forward: 5'-ACAAGCCCACTCCCCTTTAC-3' (SEQ ID NO: 38),
reverse: 5'-CAGCATAAAGTTCAACAAAGTCCC-3' (SEQ ID NO: 39)),
EIF2C2 (forward: 5'-GATCAGCATTCTTGCACTTTCTC-3' (SEQ ID NO: 40),
reverse: 5'-TCTTTCAACAGTCTATTGGGGTC-3' (SEQ ID NO: 41)),
PRKCA (forward: 5'-GCTGAAGTGTACGCCCTCTC-3' (SEQ ID NO: 42),
reverse: 5'-ACATCTTTGAGCTGTTAGGCATC-3' (SEQ ID NO: 43)), and
SESN3 (forward: 5'-AACAATGCAAAGTAGTGCTCCTC-3' (SEQ ID NO: 44),
reverse: 5'-GCTGAACTTCTTTATGCTCTTCG-3' (SEQ ID NO: 45)).

The PCR primers of other transcripts will be informed when they are requested. The conditions for PCR were; initial denaturation at 95°C for 5 min, 23 cycles (for ACTB, SNRPE, AR, PSA, NKX3.1) or 30 cycles (for TMEM46, CDKN2C, DTL, PTTG2(TBC1D1), SMC4, EIF2C2 and PRKCA) or 35 cycles (for SESN3) of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 30 sec on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized, subjected to treatment with microwave at 360 W for 1 min 4 times in antigen retrieval solution, high pH (DAKO, Carpinteria, CA), and then treated with peroxidase blocking reagent (DAKO) followed by protein block reagent (DAKO). Immunohistochemical study was carried out using the

Ventana automated IHC systems (DiscoveryTM, Ventana Medical systems, Inc., Tucson, AZ). Sections were incubated with a 1:100 diluted solution of a mouse monoclonal antibody (NCL-AR-318, NOVA CASTRA, Newcastle upon Tyne, UK) against the N-terminus portion of the human AR overnight at 4°C. The automated protocol is based on an indirect biotin-avidin system using a biotinylated universal secondary antibody and diaminobenzidine substrate with hematoxylin counterstaining.

Small interfering RNA-expressing constructs and colony formation/MTT Assay

The present inventors used small interfering RNA (siRNA)-expression vector (psiU6BX) for RNA interference effect to the target genes as described previously (Anazawa Y, *et al.* Cancer Res. 2005 Jun 1; 65(11):4578-86.). Plasmids designed to express siRNA were prepared by cloning of double-stranded oligonucleotides into psiU6BX vector. The oligonucleotide sequences of target sequences for SNRPE and ANLN are as follows: sense strand sequence for SNRPE-si1 was 5'-GGAAAGAATGAAGTGCCTT-3' (SEQ ID NO: 17; nucleotide 886-904 of SEQ ID NO: 1); for SNRPE-si3, 5'-GGTGAATGCAGAAGTGTAT-3' (SEQ ID NO: 18; nucleotide 1432-1450 of SEQ ID NO: 1), and for siANLN, 5'-CCAGTTGAGTCGACATCTG-3' (SEQ ID NO: 19; nucleotide 463-481 of SEQ ID NO: 3)). As a negative control, siEGFP: 5'-GAAGCAGCACGACTTCTTC-3' (SEQ ID NO: 20) was used. The sequences for cnstructing siRNA vector are summarize in **Table 3.**

Table 3 Sequence for siRNA

		sequence	position	SEQ ID NO
SNRPE-si1	target sequence	5'-GGAAAGAATGAAGTGCCTT-3'	886-904	17
	hairpin sequence	5'-GGAAAGAATGAAGTGCCTTTTC AAGAGAAAGGCACTTCATTCTTTC C-3'		21
	insert sequence (sense)	5'-AACAGGAAAGAATGAAGTGCCT TTTCAAGAGAAAGGCACTTCATTCT TTCC-3'		22
	insert sequence (antisense)	5'-AAAAGGAAAGAATGAAGTGCCT TTCTCTTGAAAAGGCACTTCATTCT TTCC-3'		23
SNRPE-si3	target sequence	5'-GGTGAATGCAGAAGTGTAT-3'	1432-1450	18
	hairpin sequence	5'-GGTGAATGCAGAAGTGTATTTCA AGAGAATACACTTCTGCATTCACC-3'		24

	insert sequence (sense)	5'-AACAGGTGAATGCAGAAGTGTA TTTCAAGAGAATACACTTCTGCATT CACC-3'		25
	insert sequence (antisense)	5'-AAAAGGTGAATGCAGAAGTGTA TTCTCTTGAAATACACTTCTGCATT CACC-3'		26
siANLN	target sequence	5'-CCAGTTGAGTCGACATCTG-3'	463-481	19
	hairpin sequence	5'-CCAGTTGAGTCGACATCTGTTCA AGAGACAGATGTCGACTCAACTG G-3'		27
	insert sequence (sense)	5'-AACACCAGTTGAGTCGACATCTG TTCAAGAGACAGATGTCGACTCAA CTGG-3'		28
	insert sequence (antisense)	5'-AAAACCAGTTGAGTCGACATCT GTCTCTTGAACAGATGTCGACTCA ACTGG-3'		29

Hormone-refractory prostate cancer cell lines 22Rv1 was purchased from American Type Culture Collection (ATCC, Rockville, MD), and $2x10^6$ 22Rv1 cells were grown on 10-cm dishes, transfected with psiU6-SNRPE (si1, 3) or psiU6-ANLN or psiU6-EGFP using FuGene6 reagent (Roche) according to the manufacturer's instruction, and cultured in appropriate medium containing 800 μg/ml of Geneticin for two weeks. The cells were fixed with 100 % methanol, stained with 0.1% of crystal violet-H20 for colony formation assay. In MTT assay, cell viability was measured using Cell-counting kit-8 (DOJINDO, Kumamoto, Japan) at 10 days after the transfection. Absorbance was measured at 490 nm, and at 630 nm as reference, with a Microplate Reader 550 (Bio-Rad, Hercules, CA). Preliminarily, knockdown effects of these siRNA-expression vectors on the endogenous expression of the target genes were validated 7 days after the transfection by RT-PCR using the primers described above.

Example 2: Hierarchical clustering analysis of expression profiles.

For this study, the present inventors collected 77 frozen specimens from 43 HRPC patients through prostatic needle biopsy, bone biopsy, TUR-P, or autopsy. Nearly two-thirds of specimens were not qualified for LMM and microarray analysis through the pathological evaluation by H&E staining or after evaluation of their RNA quality, and finally RNAs of only 25 HRPC specimens from 18 HRPC patients were available for further microarray analysis. All of these 18 patients had been treated with MAB (maximum androgen blockade) with LH-RH agonist treatment or surgical castration. 25 HRPC specimens included 13 HRPCs at the primary site (prostate), 8 bone metastases, 3 lymph-node metastases, and one

liver metastasis. Simultaneously, HSPC cells were microdissected from 10 untreated operable patients undergoing radical prostatectomy, and normal prostatic epithelial (NP) cells were also microdissected from five none-PC patients. These NP cells from five males were used as a normal mixture control for our cDNA microarray analysis. As shown in **Fig. 1**, the present inventors succeeded in microdissecting HRPC cells, HSPC cells and NP cells from each clinical sample to exclude the contamination of stromal cells and host organ cells at the metastatic sites (bone marrow, lymphocytes, and liver).

An unsupervised clustering analysis using expression patterns of 254 genes that the present inventors selected on the basis of strict conditions (*i.e.* valid data obtained in 80% of the experiments and expression ratios that varied by more than 1.75 SDs), clearly classified the 35 tumors into two major groups, as the HRPC and HSPC groups (**Fig. 2A**). This unsupervised clustering analysis also classified multiple tumors from the same individuals into small subgroups regardless to the metastatic organs (indicated by black boxes in **Fig. 2A**), suggesting no influence on expression patterns by host organs of their metastatic sites due to the precise microdissection technique in our laboratory.

Identification of de-regulated genes in the progression from HSPC to HRPC

To extract genes that showed significantly differential expression levels in HRPCs and HSPCs, the present inventors carried out a random permutation test using the expression profiles of 13 HRPCs at the prostate and 10 HSPCs. The present inventors selected only HRPCs at the primary site (prostate) among 25 HRPC specimens for this random permutation test because multiple HRPC samples from one individual showed quite similar patterns. The supervised clustering analysis ($P < 0.0001$, gap > 1.5) identified 36 up-regulated genes and 70 down-regulated genes in HRPCs, compared with HSPCs, which were considered to be involved in the HRPC progression and their androgen-independent and more aggressive phenotype (**Fig. 2B**). **Table 1** listed 36 up-regulated genes in HRPC, including AR (Androgen receptor), SNRPE (Small nuclear ribonucleoprotein peptide E), and ANLN (Anillin, actin binding protein). Notably, the expression level of AR in HRPC cells was much higher than that in HSPCs, which was concordant with several previous reports studying the cell line models (Gregory CW, *et al.*, Cancer Res. 1998 Dec 15;58(24):5718-24., Chen CD, *et al.*, Nat Med. 2004 Jan;10(1):33-9. Epub 2003 Dec 21., Zegarra-Moro OL, *et al.*, Cancer Res. 2002 Feb 15;62(4):1008-13.) and clinical samples (Linja MJ, *et al.*, Cancer Res. 2001 May 1;61(9):3550-5.). As shown in **Fig. 3**, semi-quantitative RT-PCR validated over-expression

of eleven genes in HRPC cells. On the other hand, **Table 2** listed 70 down-regulated genes in the progression to HRPCs, including NR4A1, CYP27A1, and HLA-A.

Table 1 Up-regulated genes in the progression to HRPC

Assignment	Accession no.	Group gap	P-value	Symbol	Gene name
1	NM_003094.2	3.306386323	3.77E-07	SNRPE	Small nuclear ribonucleoprotein polypeptide E
2	NM_018685.2	3.219579761	3.32E-06	ANLN	Anillin, actin binding protein (scraps homolog, Drosophila)
3	AA976712.1	3.182541782	5.99E-07	TMEM46	Transmembrane protein 46
4	AI357641.1	3.146687689	7.51E-06	CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
5	W67209.1	3.087139806	5.93E-08	SESN3	Sestrin 3
6	DB340904.1	2.985071675	8E-08	AR	Androgen receptor, 3'UTR
7	NM_018144.2	2.890854838	3.62E-09	SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)
8	NM_016448.1	2.811368667	7.78E-05	DTL	Denticleless homolog (Drosophila)
9	Z74616.1	2.538137903	1.92E-05	COL1A2	Collagen, type I, alpha 2
10	NM_000090.2	2.491419709	5.12E-05	COL3A1	Collagen, type III, alpha 1
11	R41754.1	2.447849423	1.19E-10	TMEM132B	Transmembrane protein 132B
12	NM_006607.1	2.440825064	5.09E-07	TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1
13	U73727.1	2.333606143	0.000024	PTPRU	Protein tyrosine phosphatase, receptor type, U
14	AK021786.1	2.319361143	3.24E-07	C17orf72	Chromosome 17 open reading frame 72
15	AA910060.1	2.236962817	8.99E-05		EST
16	AA621719.1	2.220224258	8.14E-06	SMC4	SMC4 structural maintenance of chromosomes 4-like 1
17	AK024438.1	2.198639235	7.77E-05	ZFP41	Zinc finger protein 41 homolog (mouse)
18	NM_006229.1	2.192827851	6.66E-05	PNLIPRP1	Pancreatic lipase-related protein 1

19	AA195210.1	2.170830793	2.46E-06	DKFZP761 M1511	Hypothetical protein DKFZP761M1511
20	AK096164.1	2.04647546	4.34E-06	EIF2C2	Eukaryotic translation initiation factor 2C, 2
21	AF035594.1	2.002516001	6.59E-06	PRKCA	Protein kinase C, alpha
22	NM_004442.5	1.921607125	1.06E-05	EPHB2	EPH receptor B2
23	AK096873.1	1.78604725	7.02E-05	NPEPL1	Aminopeptidase-like 1
24	NM_005733.1	1.739425422	2.56E-05	KIF20A	Kinesin family member 20A
25	AA757026.1	1.678056856	8.32E-05		EST
26	X63679.1	1.644063667	9.85E-07	TRAM1	Translocation associated membrane protein 1
27	NM_001211.4	1.636586307	9.84E-05	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta
28	NM_017915.2	1.61745888	3.12E-05	C12orf48	Chromosome 12 open reading frame 48
29	NM_014176.1	1.589009131	1.95E-08	UBE2T	Ubiquitin-conjugating enzyme E2T (putative)
30	NM_006265.1	1.56936713	1.03E-07	RAD21	RAD21 homolog (S. pombe)
31	NM_007220.3	1.563745272	-3E-13	CA5B	Carbonic anhydrase VB, mitochondrial
32	BC044310.1	1.563713145	2.78E-05	TNK2	Tyrosine kinase, non-receptor, 2
33	N51406.1	1.541763744	3.79E-05	RP11-393H10.2	Hypothetical protein FLJ14503
34	NM_005055.3	1.525830663	5.79E-05	RAPSN	Receptor-associated protein of the synapse, 43kD
35	NM_016275.3	1.519432356	1.63E-05	SELT	Selenoprotein T
36	NM_006988.3	1.504404832	6.73E-05	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1

Table 2 Down-regulated genes in the progression to HRPC

Assignment	Accession no.	Group gap	P-value	Symbol	Gene name
37	BC092418.1	5.558869662	1.22E-06	MYBPC1	Myosin binding protein C, slow type
38	X04325.1	5.062445046	1.06E-05	GJB1	Gap junction protein, beta 1, 32kDa

39	NM_001150.1	4.826495535	7.40E-10	ANPEP	Alanyl (membrane) aminopeptidase
40	NM_024080.3	4.090779772	9.33E-12	TRPM8	Transient receptor potential cation channel, subfamily M member 8
41	NM_002443.2	3.771232027	4.03E-09	MSMB	Microseminoprotein, beta-
42	L13740.1	3.204461772	7.41E-35	NR4A1	Nuclear receptor subfamily 4, group A, member 1
43	NM_000784.2	3.176792485	2.03E-12	CYP27A1	Cytochrome P450, family 27, subfamily A, polypeptide 1
44	AA243967.1	3.119889956	3.91E-05	ACPP	Acid phosphatase, prostate
45	NM_138342.2	3.049236542	5.45E-05	LOC89944	Hypothetical protein BC008326
46	AF266280.1	2.994099827	7.11E-06	LGALS3	Lectin, galactoside-binding, soluble, 3 (galectin 3)
47	AB010419.1	2.95051338	2.21E-05	CBFA2T3	Core-binding factor, runt domain, alpha subunit 2; translocated 3
48	NM_003407.1	2.906880911	1.24E-09	ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)
49	NM_032801.3	2.863996452	1.93E-08	JAM3	Junctional adhesion molecule 3
50	NM_004417.2	2.840006269	2.20E-10	DUSP1	Dual specificity phosphatase 1
51	NM_005252.2	2.779578672	2.58E-06	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
52	Y11339.2	2.673481738	1.55E-13	ST6GALNAC1	ST6-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
53	L19871.1	2.519745453	1.64E-09	ATF3	Activating transcription factor 3
54	BC016952.1	2.420887227	4.47E-10	CYR61	Cysteine-rich, angiogenic inducer, 61
55	N70019.1	2.393780488	1.28E-05	MT1M	Metallothionein 1M
56	NM_005139.2	2.30234995	7.04E-20	ANXA3	Annexin A3
57	NM_005767.3	2.292587029	2.07E-05	P2RY5	Purinergic receptor P2Y, G-protein coupled, 5
58	M62829.1	2.268933663	5.38E-05	EGR1	Early growth response 1

59	R38989.1	2.188002228	8.97E-08	SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2
60	NM_001669.2	2.186821444	5.88E-11	ARSD	Arylsulfatase D
61	NM_001584.1	2.17939094	3.05E-06	MPPED2	Metallophosphoestera se domain containing 2
62	L02950.1	2.16892321	1.08E-09	CRYM	Crystallin, mu
63	NM_032592.1	2.148649429	3.29E-05	PHACS	1-aminocyclopropane-1-carboxylate synthase
64	NM_015267.1	2.123686657	6.64E-07	CUTL2	Cut-like 2 (Drosophila)
65	R42862.1	2.085435644	2.09E-06		EST
66	NM_014861.1	2.024649382	7.07E-09	KIAA0703	KIAA0703 gene product
67	AL832642.2	2.022123591	5.38E-06	CD44	CD44 molecule (Indian blood group)
68	NM_005891.1	2.020215882	8.92E-11	ACAT2	Acetyl-Coenzyme A acetyltransferase 2
69	AF070632.1	2.020131066	2.51E-15		EST
70	AA742701.1	2.017839777	4.39E-10	LCP1	Lymphocyte cytosolic protein 1 (L-plastin)
71	NM_014841.1	2.007560931	5.59E-05	SNAP91	Synaptosomal-associated protein, 91kDa homolog (mouse)
72	M62831.1	2.005374607	3.91E-11	IER2	Immediate early response 2
73	NM_178835.2	1.975475229	3.50E-05	LOC152485	Hypothetical protein LOC152485
74	NM_024709.2	1.96745649	3.60E-05	C1orf115	Chromosome 1 open reading frame 115
75	DA313595.1	1.961835426	2.05E-05	HLA-A	Major histocompatibility complex, class I, A
76	X04481.1	1.915026305	6.40E-06	C2	Complement component 2
77	NM_173653.1	1.912986031	2.69E-09	SLC9A9	Solute carrier family 9 (sodium/hydrogen exchanger), member 9
78	NM_002228.3	1.872688976	3.02E-05	JUN	V-jun sarcoma virus 17 oncogene homolog (avian)
79	X07549.1	1.852416069	1.51E-07	CTSH	Cathepsin H
80	L05779.1	1.835437702	7.24E-08	EPHX2	Epoxide hydrolase 2, cytoplasmic
81	BC012037.1	1.832746195	5.02E-06	NBL1	Neuroblastoma, suppression of tumorigenicity 1

82	L13288.1	1.830209324	1.63E-08	VIPR1	Vasoactive intestinal peptide receptor 1
83	NM_004842.2	1.817118309	7.01E-12	AKAP7	A kinase (PRKA) anchor protein 7
84	NM_016573.2	1.813774769	1.80E-11	GMIP	GEM interacting protein
85	AF237813.1	1.811745708	1.59E-08	ABAT	4-aminobutyrate aminotransferase
86	U47025.1	1.752036006	3.21E-08	PYGB	Phosphorylase, glycogen; brain
87	NM_005080.2	1.745150156	1.13E-08	XBP1	X-box binding protein 1
88	BQ182018.1	1.709983737	1.27E-07	SSU72	SSU72 RNA polymerase II CTD phosphatase homolog
89	AK129574.1	1.702855987	1.32E-05	DOCK5	Dedicator of cytokinesis 5
90	AK026400.1	1.691754511	1.85E-07	FLJ42562	Similar to echinoderm microtubule associated protein like 5
91	X51345.1	1.688349706	1.63E-09	JUNB	Jun B proto-oncogene
92	NM_014010.3	1.66324122	6.53E-05	ASTN2	Astrotactin 2
93	NM_000527.2	1.647252497	1.35E-05	LDLR	Low density lipoprotein receptor (familial hypercholesterolemia)
94	NM_014174.2	1.645153682	1.13E-05	THYN1	Thymocyte nuclear protein 1
95	AA523303.1	1.63829997	2.70E-08	DEF6	Differentially expressed in FDCP 6 homolog (mouse)
96	X12548.1	1.620216872	1.61E-08	ACP2	Acid phosphatase 2, lysosomal
97	BX648582.1	1.611126501	5.56E-09	SPRY2	Sprouty homolog 2 (Drosophila)
98	NM_005951.1	1.598742883	5.62E-08	MT1H	Metallothionein 1H
99	NM_014553.1	1.594509479	8.36E-05	TFCP2L1	Transcription factor CP2-like 1
100	M96824.1	1.593941987	1.88E-09	NUCB1	Nucleobindin 1
101	U79240.1	1.567475917	5.60E-08	PASK	PAS domain containing serine/threonine kinase
102	NM_006633.1	1.564791961	7.99E-05	IQGAP2	IQ motif containing GTPase activating protein 2
103	NM_017679.2	1.547638171	5.30E-09	BCAS3	Breast carcinoma amplified sequence 3
104	NM_000295.3	1.545151445	6.49E-06	SERPINA1	Serpin peptidase inhibitor, clade A, member 1

105	AL390079.1	1.52908521	4.32E-08	LOC58489	Hypothetical protein from EUROIMAGE 588495
106	NM_000282.2	1.527984534	3.77E-10	PCCA	Propionyl Coenzyme A carboxylase, alpha polypeptide

Example 3: AR expression and activity in clinical HRPC cells.

The present inventors further analyzed AR protein expression in clinical HRPCs by immunohistochemistry and the expression level of several AR-regulated genes, which should reflect the actual AR activity as a transcriptional factor in the nucleus. Immunohistochemical analysis for AR using 6 HRPCs and 16 HSPCs showed the positive staining in the nuclear of all HRPC, HSPC, and normal prostate. AR protein in the nucleus was expressed in similar levels in HRPCs (**Fig. 4A**), HSPCs (**Fig. 4B**), and normal prostate (**Fig. 4C**), although the RNA expression level of AR was much higher in HRPC cells than in HSPC and NP cells (**Fig. 3** and **Fig. 4D**). Furthermore, RNA expression levels of AR-regulated genes, PSA and NKX3.1 (Masuda K, *et al.*, J Mol Biol. 2005 Nov 4; 353(4):763-71. Epub 2005 Sep 22.), in HRPC cells were also very similar to those in HSPC and NP cells (**Fig. 4D**), concordant with AR protein levels in the nucleus. These findings implicated that clinical HRPC cells are likely to maintain AR activity in the cell by overexpressing AR mRNA, under very low level of circulating testicular androgen, but stabilized AR protein and actual AR activity levels as a transcriptional factor in the nucleus of HRPC cells was similar to that in HSPC cells and NP cells.

Example 4: Knockdown effect of ANLN and SNRPE on HRPC cell growth

To investigate the contribution of non-AR pathways or genes to HRPC phenotype, the present inventors selected ANLN and SNRPE, which showed a significantly high level of expression in HRPCs (**Fig. 3**, and **Table 1**). The present inventors constructed several vectors designed to express siRNA specific to ANLN (siANLN) and SNRPE (si1 and si3), and transfected each of them into HRPC cell line 22Rv1. The transfection with siANLN showed the significant knockdown effect on the ANLN transcript (**Fig. 5A**), and resulted in drastic reduction of the numbers of colonies (**Fig. 5B**) as well as those of the viable cells measured by MTT assay (**Fig. 5C**), while the transfection of a negative control (siEGFP) did not affect. Among three siRNA constructs to SNRPE, SNRPE-si1 and SNRPE-si3 significantly knocked down the transcription level of SNRPE (**Fig. 5D**) and caused drastic reduction of the numbers of colonies (**Fig. 5E**) as well as those of the viable cells measured by MTT assay (**Fig. 5F**),

while the transfection of a negative control (siEGFP) showed no or little knockdown effect on SNRPE expression and did not affect cell viability. These findings suggested that a non-AR pathway(s) represented by over-expressing genes in HRPC such as SNRPE and ANLN could play important roles in the cell viability of HRPC in addition to the AR pathway.

Discussion

Most of the patients with relapsed or advanced PC respond well to androgen-ablation therapy. However, the tumors eventually acquire androgen-independent and more aggressive phenotype for which no effective anti-cancer drug or therapy is available at present. In this invention, the molecular mechanisms of acquirement of this more aggressive phenotype were approached by analyzing human HRPC clinical samples, but not by the use of cell lines or mouse models. The gene expression studies for clinical HRPCs have been very limited so far, largely due to difficulties to obtain appropriate frozen HRPC samples (Shah RB, *et al.* Cancer Res. 2004 Dec 15;64(24):9209-16., Stanbrough M, *et al.* Cancer Res. 2006 Mar 1;66(5):2815-25.). Hence, the present inventors are confident that our precise genome-wide expression profiles of clinical HRPC cells are very valuable. The random permutation test comparing the expression profiles of 13 HRPCs at the prostate with those of 10 HSPCs identified 36 up-regulated genes and 70 down-regulated genes in HRPCs (**Tables 1** and **2**). Some of such genes were considered to be associated with androgen-independent and more aggressive phenotypes of clinical HRPCs. Among the 36 up-regulated genes in HRPCs, first, the present inventors focused on AR over-expression. In spite of AR transactivation of mRNA in HRPC cells, the amount of stabilized AR protein in the nucleus and AR activity measured by the expression levels of its down-stream target genes in HRPC cells were very similar to those in HSPC and normal prostate epithelial cells. Several reports suggested that even under low level of circulating testicular androgen, HRPCs still maintain some level of dependency to the AR pathway (Chen CD, *et al.* Nat Med. 2004 Jan;10(1):33-9. Epub 2003 Dec 21., Zegarra-Moro OL, *et al.*, Cancer Res. 2002 Feb 15;62(4):1008-13., Stanbrough M, *et al.*, Cancer Res. 2006 Mar 1;66(5):2815-25., Mohler JL, *et al.*, Clin Cancer Res. 2004 Jan 15;10(2):440-8.) and our data also support this idea. However, of course, the retention of the AR activity itself does not explain the more aggressive phenotype of clinical HRPCs, and apparently the non-AR pathway(s) should contribute to this clinical HRPC phenotype.

The up-regulated genes in HRPC included ANLN and SNRPE as well as AR. ANLN (Anillin, actin-binding protein) interacts with and activated RhoA, and that this

complex is likely to be essential for the growth-promoting pathway and aggressive features of lung cancers through PI3K/Akt signaling (Suzuki C, *et al.*, Cancer Res 2005; 65:11314-25.), indicating that its over-expression in HRPCs can be involved with aggressive phenotype of clinical HRPCs. SNRPE (small nuclear ribonucleoprotein peptide E) may be involved with RNA splicing, but its function is unknown. Our siRNA experiments demonstrated that over-expression of ANLN and SNRPE could play some important roles in the viability of HRPC cells.

The list of the down-regulated genes in HRPC includes NR4A1, CYP27A1, and HLA-A antigen. NR4A1 belongs to the steroid nuclear hormone receptor superfamily and its expression can cause apoptosis (Woronicz JD, *et al.*, Nature. 1994 Jan 20;367(6460):277-81.). NR4A1 expression is regulated by LH (luteinizing hormone) (Song KH, *et al.*, Endocrinology. 2001 Dec;142(12):5116-23.) and its down-regulation in HRPCs can reflect LH depletion in the patients under the treatment of LH-RH antagonist. CYP27A1 catalyses hydroxylations in the bioactivation of vitamin D3 (Tokar EJ & Webber MM. Clin Exp Metastasis. 2005;22(3):275-84.). Epidemiological evidence suggests an inverse relationship between prostate cancer and serum vitamin D levels (Hanchette CL & Schwartz GG. Cancer. 1992 Dec 15; 70(12):2861-9.), and active vitamin D3 inhibits growth and invasion of human prostate cancer cells (Tokar EJ & Webber MM. Clin Exp Metastasis. 2005; 22(3):275-84.). Down-regulation of NR4A1 and CYP27A1 can provide HRPC cells with some advantages for their survival and growth. Notably, HLA-A antigen, one of the major histocompatibility complex (MHC) molecules, and many other HLA antigens (which were not listed in **Table2** because of their P value = 0.001-0.0001) were significantly down-regulated in clinical HRPCs, implicating HRPC cells could acquire immuno-tolerance (Lu QL, *et al.*, J Pathol. 2000 Feb; 190(2):169-76.).

The present inventors attempted to identify the genes that were differentially expressed between HRPCs in metastatic site and those in the primary site. Because prostate cancer can preferentially metastasize to bone, and a number of reports (Chung LW. Cancer. 2003 Feb 1;97(3 Suppl):772-8., Cher ML, *et al.*, Am J Pathol. 2006 May;168(5):1405-12.) suggested that the microenvironment in bone marrow could promote prostate cancer growth and change their phenotype more aggressive. In comparing the gene expression patterns between metastatic tumors and primary tumors, it is critical to exclude the cells of the host organs of the metastatic tumors, and the expression profiles of bone metastasis of PC was

very vulnerable to contamination of bone marrow cells (Stanbrough M, *et al.*, Cancer Res. 2006 Mar 1;66(5):2815-25.). In our study, the expression profiles of the microdissected cancer cells in bone metastasis was expected to reflect such inferences with the microenvironment in bone marrow and the present inventors performed the supervised clustering analysis using the expression profiles of 8 HRPC cells at bone metastatic lesions and 10 HRPC cells at the prostate. However, our analysis failed to distinguish them. Taken together with the unsupervised analysis, our data unexpectedly indicated that the differences in expression patterns among the multiple metastatic loci derived from the individual patients were much smaller than the inter-individual differences in the expression patterns.

In conclusion, our precise microarray analysis of HRPC cells provides useful information to understand the molecular mechanism of HRPC progression and HRPC phenotype, and to identify molecular targets for the treatment of HRPC.

INDUSTRIAL APPLICABILITY

The gene-expression analysis of HRPC and HSPC described herein, obtained through a combination of laser-capture microdissection and genome-wide cDNA microarray, has identified specific genes as targets for cancer prevention and therapy. Based on the expression of a subset of these differentially expressed genes, the present invention provides molecular diagnostic markers for diagnosing or testing HRPC.

The methods described herein are also useful in the identification of additional molecular targets for prevention, and treatment of HRPC. The data reported herein add to a comprehensive understanding of HRPC, facilitate development of novel diagnostic strategies, and provide clues for identification of molecular targets for therapeutic drugs and preventative agents. Such information contributes to a more profound understanding of prostatic tumorigenesis, and provides indicators for developing novel strategies for diagnosis, treatment, and ultimately prevention of HRPC.

For instance, transition from HSPC to HRPC can be monitored by the present invention. Alternatively, the presence of HRPC cells in HSPC tissues can also be detected by the present invention. Hormone therapies such as androgen-ablation therapy are generally effective for treating prostate cancer. However, during hormone therapy, progression from HSPC to HRPC is frequently observed. Once the progression occurred, it is necessary to

immediately change the therapeutic strategy to something other than hormone therapy, as soon as possible. The present invention provides methods for monitoring the progression of HSPC to HRPC, and contributes to the selection of a suitable therapeutic strategy for HRPC at an early stage.

All patents, patent applications, and publications cited herein are incorporated by reference in their entirety. Furthermore, while the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

CLAIMS

1. A method of diagnosing hormone-refractory prostate cancer (HRPC) in a subject, comprising determining an expression level of an HRPC-associated gene in a patient-derived biological sample, wherein an increase or decrease of said level compared to expression level of said gene in hormone-sensitive prostate cancer indicates that said subject has hormone-refractory prostate cancer.

2. The method of claim 1, wherein said HRPC-associated gene is selected from the group consisting of HRPC 1-36, wherein an increase in said level compared to a level in hormone-sensitive prostate cancer indicates said subject has hormone-refractory prostate cancer.

3. The method of claim 2, wherein said increase is at least 10% greater than said level in hormone-sensitive prostate cancer.

4. The method of claim 1, wherein said HRPC-associated gene is selected from the group consisting of HRPC 37-106, wherein a decrease in said level compared to a level in hormone-sensitive prostate cancer indicates said subject has hormone-refractory prostate cancer.

5. The method of claim 4, wherein said decrease is at least 10% lower than said level in hormone-sensitive prostate cancer.

6. The method of claim 1, wherein said method further comprises determining the expression level of a plurality of HRPC-associated genes.

7. The method of claim 1, wherein the expression level is determined by any one method selected from the group consisting of:
   a) detecting the mRNA of the HRPC-associated gene;
   b) detecting the protein encoded by the HRPC-associated gene; and
   c) detecting the biological activity of the protein encoded by the HRPC-associated gene.

8. The method of claim 1, wherein said expression level is determined by detecting hybridization of an HRPC-associated gene probe to a gene transcript of said patient-derived biological sample.

9. The method of claim 8, wherein said hybridization step is carried out on a DNA array.

10. The method of claim 1, wherein said biological sample comprises prostate cells.

11. The method of claim 10, wherein said prostate cells obtained from cancerous prostate tissue.

12. The method of claim 11, wherein said cancerous prostate tissue comprises HSPC cells.

13. A hormone-refractory prostate cancer reference expression profile, comprising a pattern of gene expression of two or more genes selected from the group consisting of HRPC 1-106.

14. A hormone-refractory prostate cancer reference expression profile, comprising a pattern of gene expression of two or more genes selected from the group consisting of HRPC 1-36.

15. A hormone-refractory prostate cancer reference expression profile, comprising a pattern of gene expression of two or more genes selected from the group consisting of HRPC 37-106.

16. A method of screening for a compound for treating or preventing hormone-refractory prostate cancer, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by any one of HRPC 1-106;

b) detecting the binding activity between the polypeptide and the test compound; and

c) selecting a compound that binds to the polypeptide.

17. A method of screening for a compound for treating or preventing hormone-refractory prostate cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell expressing one or more marker genes, wherein the one or more marker genes is selected from the group consisting of HRPC 1-106; and

b) selecting a compound that reduces the expression level of one or more marker genes selected from the group consisting of HRPC 1-36, or elevates the expression level of one or more marker genes selected from the group consisting of HRPC 37-106.

18. The method of claim 17, wherein said test cell comprises a prostate cancer cell.

19. A method of screening for a compound for treating or preventing hormone-refractory prostate cancer, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by any one of HRPC 1-106;

b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by HRPC 1-36 in comparison with the biological activity detected in the absence of the test compound, or enhances the biological activity of the polypeptide encoded by HRPC 37-106 in comparison with the biological activity detected in the absence of the test compound.

20. A method of screening for a compound for treating or preventing hormone-refractory prostate cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of one or more marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the one or more marker genes are selected from the group consisting of HRPC 1-106;

b) measuring the expression level or activity of said reporter gene; and

c) selecting a compound that reduces the expression level or activity of said reporter gene when said marker gene is an up-regulated marker gene selected from the group consisting of HRPC 1-36 as compared to a level in control, or that enhances the expression level of said reporter gene when said marker gene is a down-regulated marker gene selected from the group consisting of HRPC 37-106 as compared to a level in control.

21. A kit comprising one or more detection reagents which each bind to a nucleic acid sequence selected from the group consisting of HRPC 1-106.

22. An array comprising a plurality of nucleic acids which each bind to a nucleic acid sequence selected from the group consisting of HRPC 1-106.

23. A method of treating or preventing hormone-refractory prostate cancer in a subject comprising administering to said subject an antisense composition, said composition

comprising a nucleotide sequence complementary to a coding sequence selected from the group consisting of HRPC 1-36.

24. A method of treating or preventing hormone-refractory prostate cancer in a subject comprising administering to said subject a double-stranded molecule composition, wherein said composition reduces the expression of a nucleic acid sequence selected from the group consisting of HRPC 1-36.

25. The method of claim 24, wherein the nucleic acid sequence is either or both of HRPC 1 (SNRPE) and HRPC 2 (ANLN).

26. The method of claim 25, wherein the double-stranded molecule composition comprises

(i) a sense strand which comprises a nucleotide sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs; 17, 18, and 19, and

(ii) an antisense strand which comprises a nucleotide sequence complementary to said sense strand.

27. A method of treating or preventing hormone-refractory prostate cancer in a subject comprising the step of administering to said subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of HRPC 1-36.

28. A method of treating or preventing hormone-refractory prostate cancer in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid selected from the group consisting of HRPC 1-36 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide.

29. A method of treating or preventing hormone-refractory prostate cancer in a subject comprising administering to said subject a compound that increases the expression or activity of HRPC 37-106.

30. A method of treating or preventing hormone-refractory prostate cancer in a subject comprising administering to said subject a pharmaceutically effective amount of

polynucleotide selected from the group consisting of HRPC 37-106, or polypeptide encoded by thereof.

31. A composition for treating or preventing hormone-refractory prostate cancer, said composition comprising a pharmaceutically effective amount of an antisense polynucleotide or a double-stranded molecule against a polynucleotide selected from the group consisting of HRPC 1-36 as an active ingredient, and a pharmaceutically acceptable carrier.

32. The composition of claim 30, wherein the polynucleotide is either or both of HRPC 1 (SNRPE) and HRPC 2 (ANLN).

33. The composition of claim 32, wherein the double-stranded molecule comprises

(i) a sense strand which comprises a nucleotide sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs; 17, 18, and 19, and

(ii) an antisense strand which comprises a nucleotide sequence complementary to said sense strand.

34. A composition for treating or preventing hormone-refractory prostate cancer, said composition comprising a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of HRPC 1-36 as an active ingredient, and a pharmaceutically acceptable carrier.

35. A double-stranded molecule comprising a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs: 17 and 18, and wherein the antisense strand comprises a nucleotide sequence which is complementary to said sense strand, wherein said sense strand and said antisense strand hybridize to each other to form said double-stranded molecule, and wherein said double-stranded molecule, when introduced into a cell expressing the SNRPE gene, inhibits expression of said gene.

36. The double-stranded molecule of claim 35, wherein said double-stranded molecule is a single nucleotide transcript comprising the sense strand and the antisense strand linked

via a single-stranded nucleotide sequence.

37. The double-stranded molecule of claim 35, wherein the double stranded molecule is an oligonucleotide of between about 19 and about 25 nucleotides in length.

38. A vector encoding the double-stranded molecule of claim 35.

39. The vector of claim 38, wherein the vector encodes a transcript having a secondary structure and comprises the sense strand and the antisense strand.

40. The vector of claim 39, wherein the transcript further comprises a single-stranded nucleotide sequence linking said sense strand and said antisense strand.

41. A vector comprising a polynucleotide comprising a combination of a sense strand nucleic acid and an antisense strand nucleic acid, wherein said sense strand nucleic acid comprises nucleotide sequence of SEQ ID NOs: 17 or 18, and said antisense strand nucleic acid consists of a sequence complementary to the sense strand.

42. The vector of claim 41, wherein said polynucleotide has the general formula
5'-[A]-[B]-[A']-3'
wherein [A] is a nucleotide sequence of SEQ ID NOs: 17 or 18; [B] is a nucleotide sequence consisting of 3 to 23 nucleotides; and [A'] is a nucleotide sequence complementary to [A].

****

Fig. 1

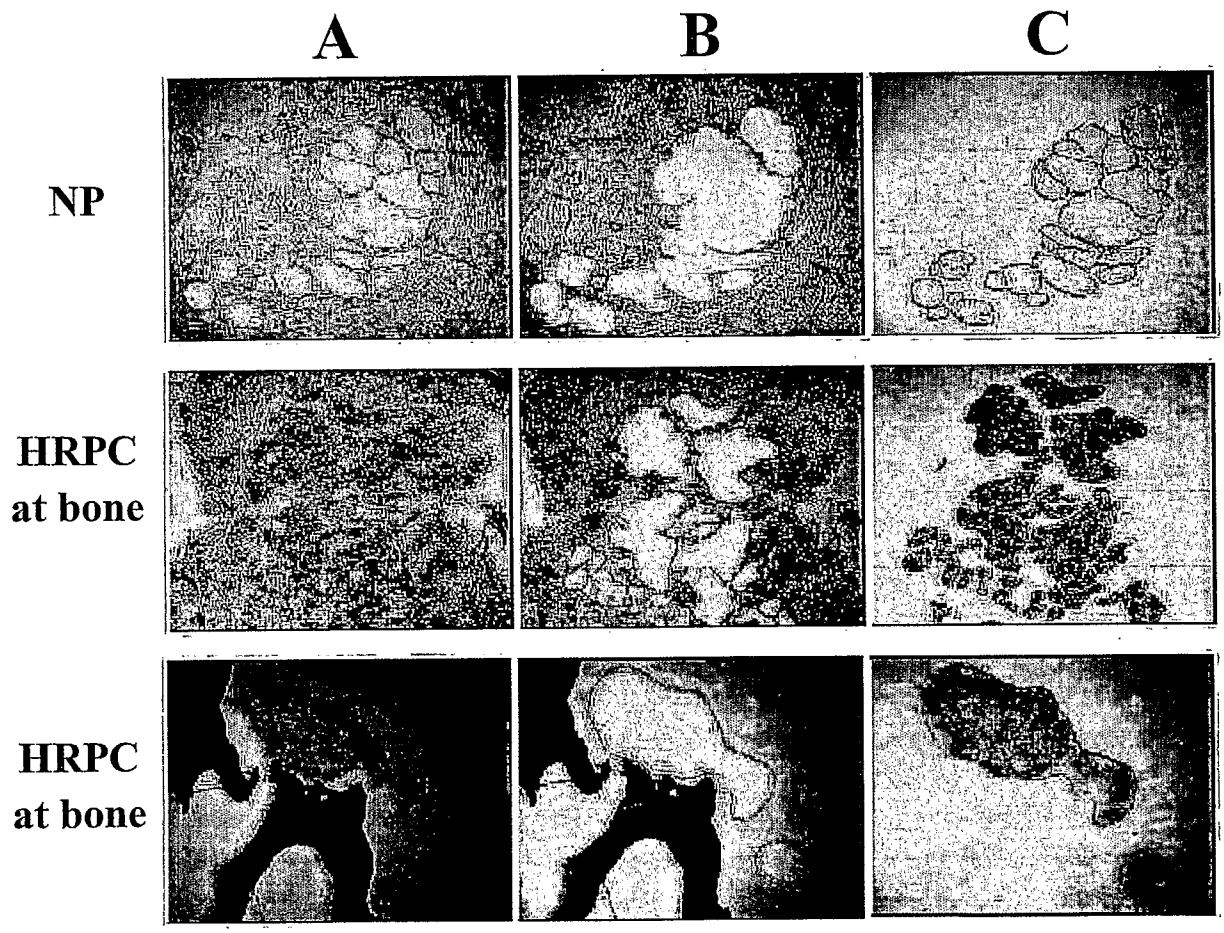


Fig. 2

(A)

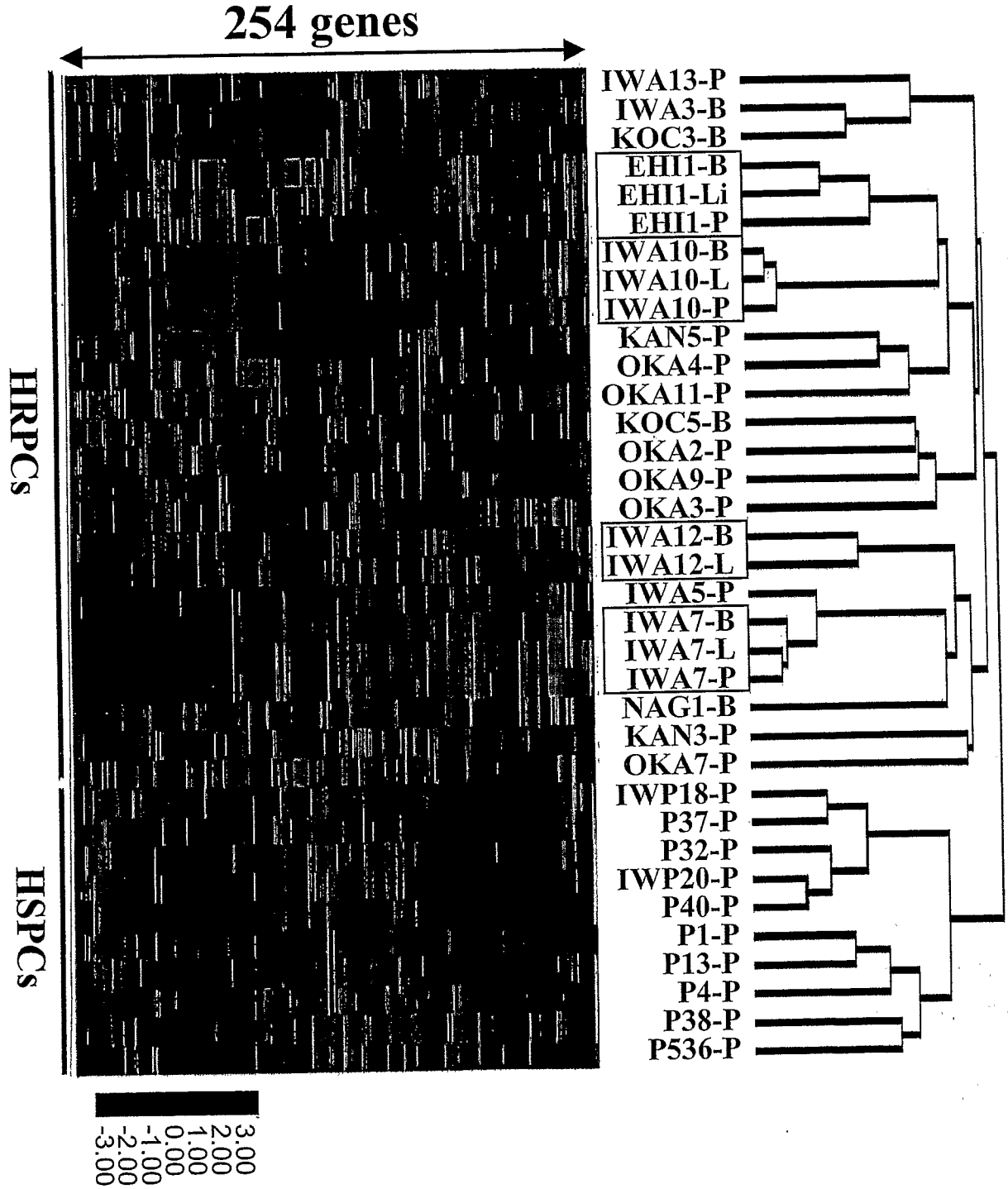

254 genes
HRPCs
HSPCs
IWA13-P
IWA3-B
KOC3-B
EHI1-B
EHI1-Li
EHI1-P
IWA10-B
IWA10-L
IWA10-P
KAN5-P
OKA4-P
OKA11-P
KOC5-B
OKA2-P
OKA9-P
OKA3-P
IWA12-B
IWA12-L
IWA5-P
IWA7-B
IWA7-L
IWA7-P
NAG1-B
KAN3-P
OKA7-P
IWP18-P
P37-P
P32-P
IWP20-P
P40-P
P1-P
P13-P
P4-P
P38-P
P536-P
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00

Fig. 2

(B)

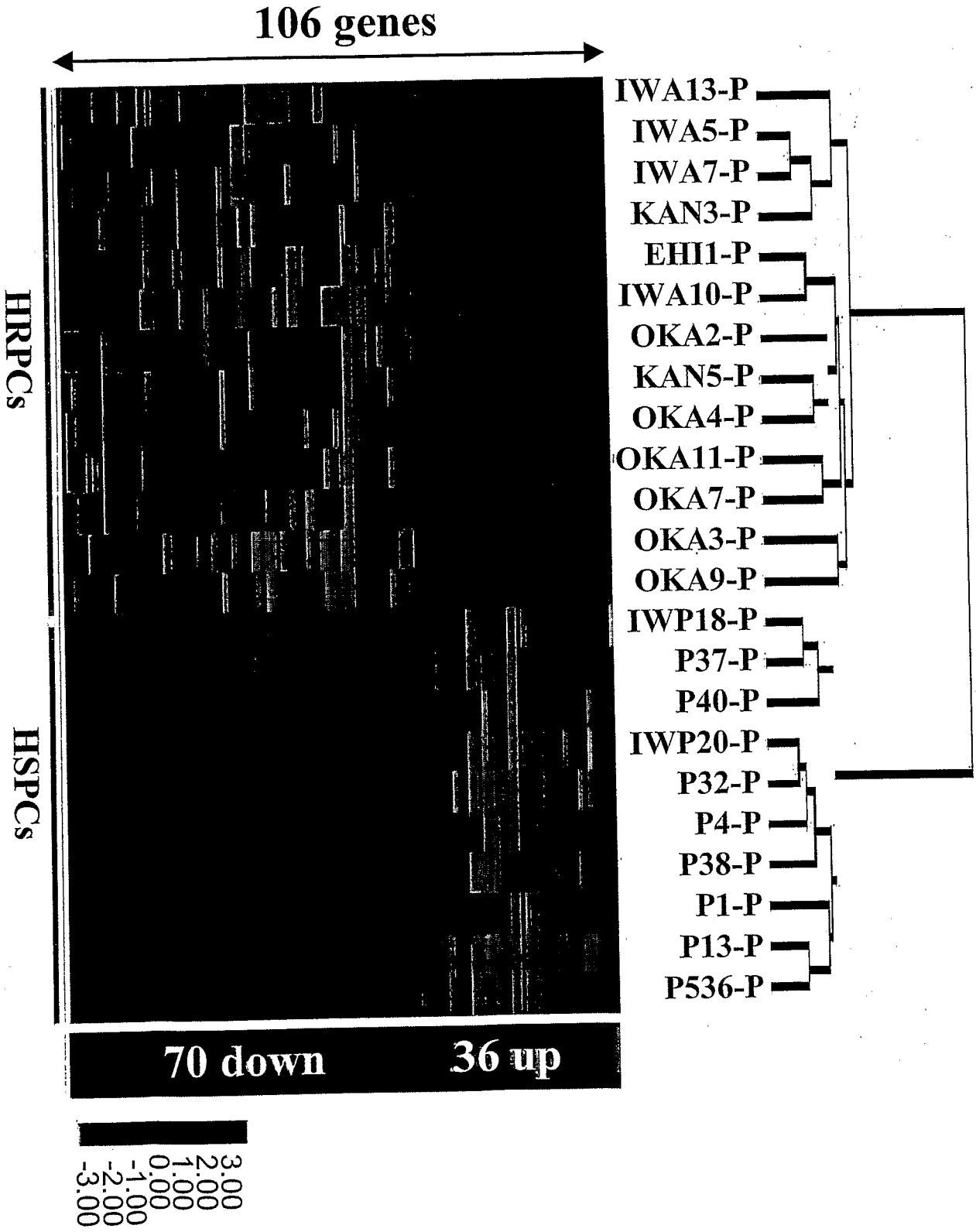

106 genes
HRPCs
HSPCs
IWA13-P
IWA5-P
IWA7-P
KAN3-P
EHI1-P
IWA10-P
OKA2-P
KAN5-P
OKA4-P
OKA11-P
OKA7-P
OKA3-P
OKA9-P
IWP18-P
P37-P
P40-P
IWP20-P
P32-P
P4-P
P38-P
P1-P
P13-P
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1.00
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-1.00
-2.00
-3.00

Fig. 3

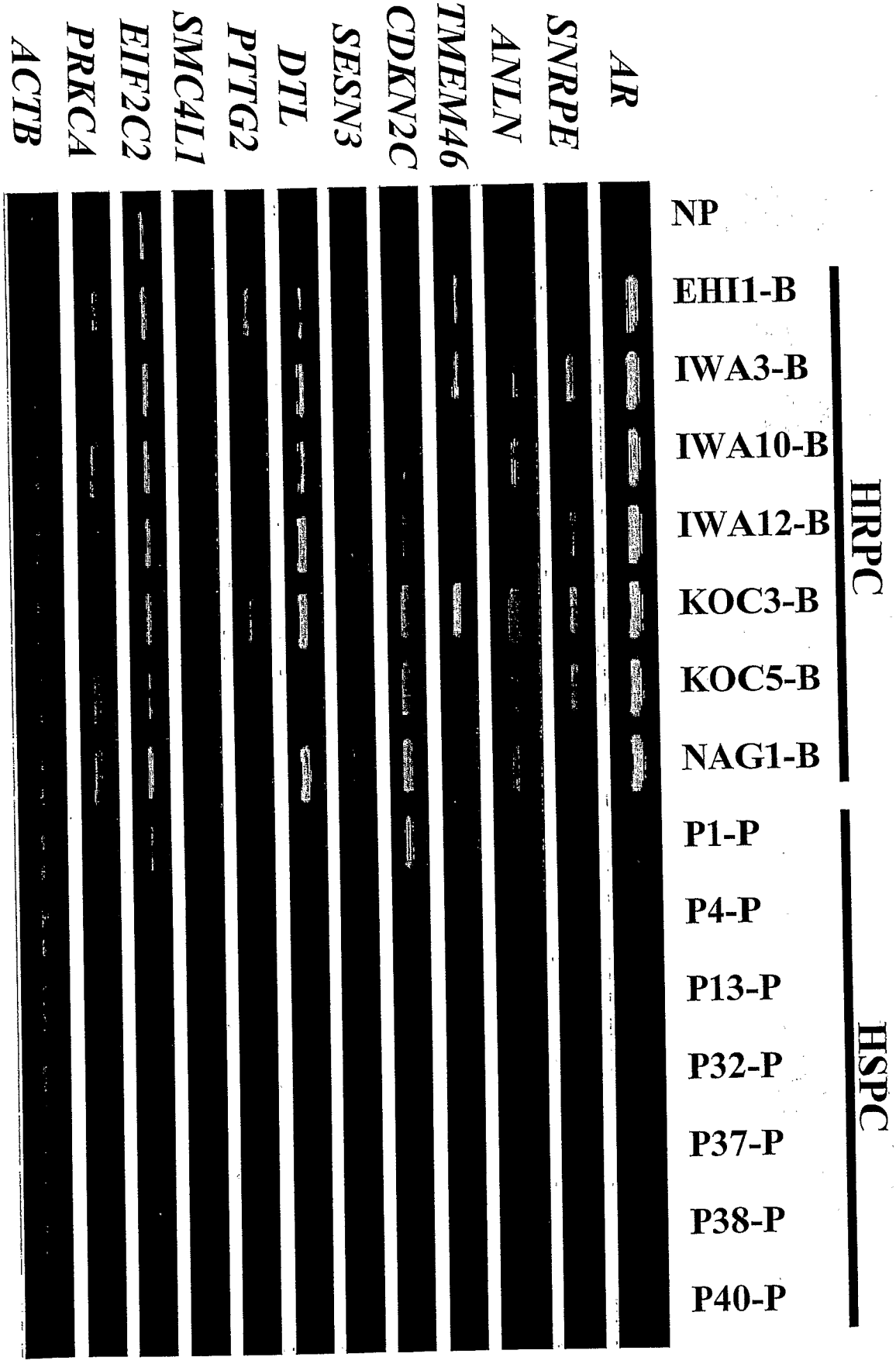

AR
SNRPE
ANLN
TMEM46
CDKN2C
SESN3
DTL
PTTG2
SMC4L1
EIF2C2
PRKCA
ACTB
NP
EHI1-B
IWA3-B
IWA10-B
IWA12-B
KOC3-B
KOC5-B
NAG1-B
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P40-P
HRPC
HSPC

Fig. 4

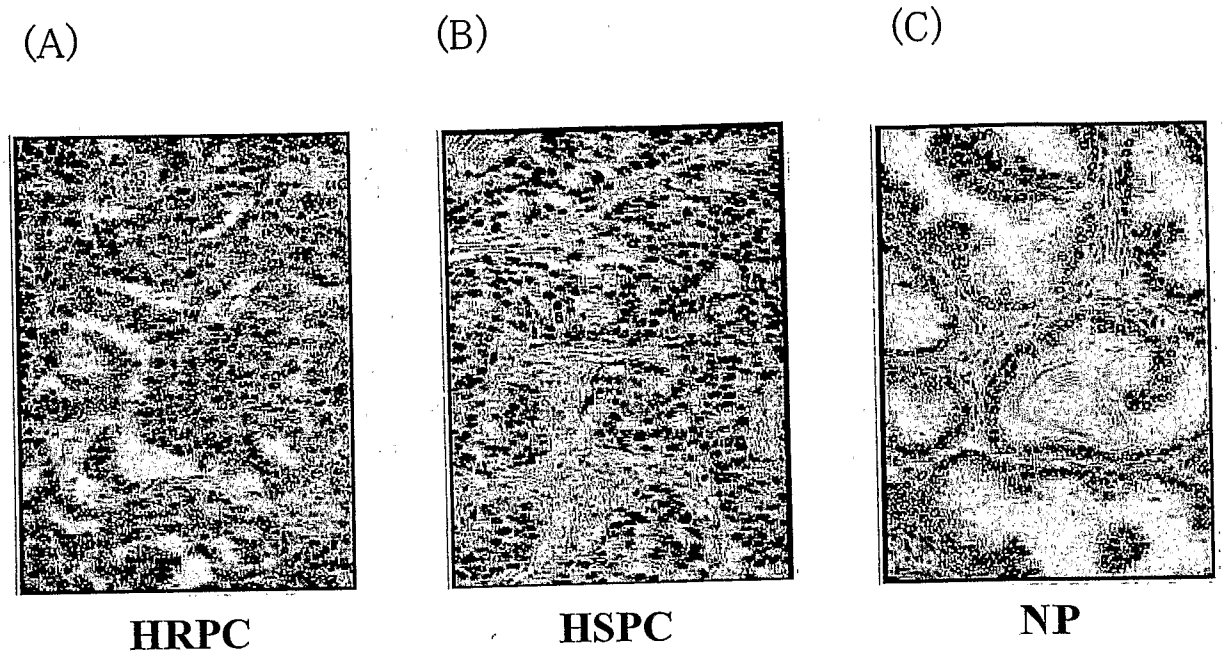
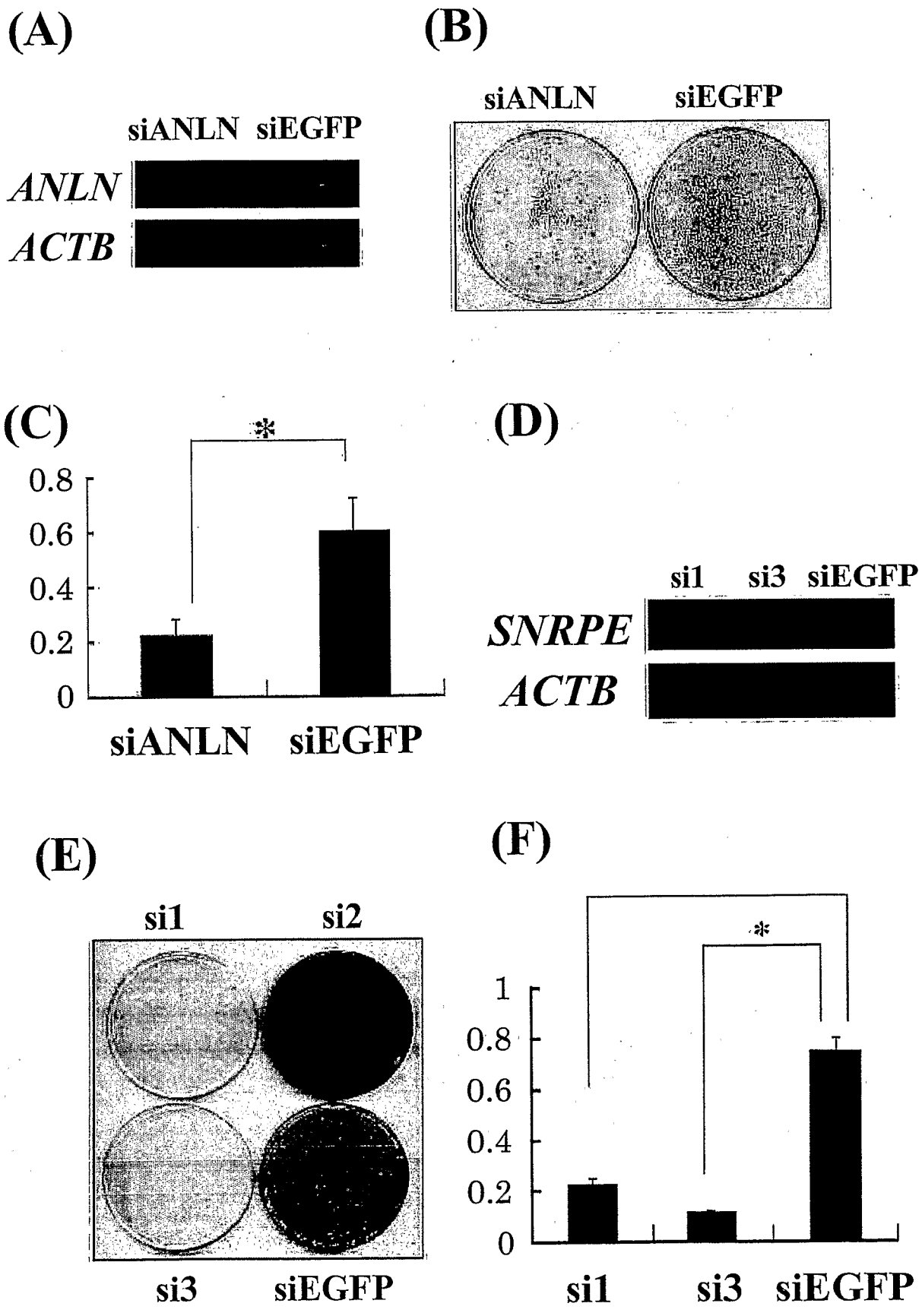

(A)
(B)
(C)
HRPC
HSPC
NP
(D)
HRPC
HSPC
NP
EHI1-B
IWA3-B
IWA10-B
IWA12-B
KOC3-B
KOC5-B
NAG1-B
P1-P
P4-P
P13-P
P32-P
P37-P
P38-P
P40-P
AR
PSA
NKX3.1
ACTB

Fig. 5


(A)
siANLN siEGFP
ANLN
ACTB
(B)
siANLN siEGFP
(C)
*
0.8
0.6
0.4
0.2
0
siANLN siEGFP
(D)
si1 si3 siEGFP
SNRPE
ACTB
(E)
si1 si2
si3 siEGFP
(F)
*
1
0.8
0.6
0.4
0.2
0
si1 si3 siEGFP

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/053133

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N15/09(2006.01)i, C12Q1/68(2006.01)i, G01N33/50(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N15/09, C12Q1/68, G01N33/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2008
Registered utility model specifications of Japan 1996-2008
Published registered utility model applications of Japan 1994-2008

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS/WPI(DIALOG), GenBank/EMBL/DDBJ/GeneSeq, SwissProt/PIR/Geneseq , JSTPlus(JDream2), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2002/022642 A1 (The University of British Colombia) 2002.03.21, Example 2 & JP 2004-532794 A & US 2004/0072776 A1 & EP 1317470 A1	1-22,31-42
Y	JP 2004-059502 A (Takeda Chemical Industries, LTD.) 2004.02.26, 【0002】～【0004】 (Family:none)	1-22,31-42
Y	WO 2005/083118 A2 (Oncotherapy Science, Inc.) 2005.09.09, All documents & JP 2007-525220 A & EP 1733047 A1 & EP 1730279 A1	1-22,31-42

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11.03.2008	25.03.2008

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/053133

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2005/090398 A1 (Oncotherapy Science, INC.) 2005.09.29, all documents & JP 2008-505601 A & EP 1727831 A1	1-22,31-42
Y	WO 2004/091548 A2 (Avalon Pharmaceuticals, INC.) 2004.10.28, all documents & JP 2006-523456 A & EP 1620573 A1 & US 2007/0059697 A1	1-22,31-42
Y	Database GenBank [online], Accessin No. Z74616, <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1418929> 14-Nov-2006 uploaded, [retrieved on 2008-03-011] Definition: H.sapiens mRNA for prepro-alpha2(I) collagen.	1-22,31-42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/053133

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

a. type of material

- [x] a sequence listing
- [ ] table(s) related to the sequence listing

b. format of material

- [ ] on paper
- [x] in electronic form

c. time of filing/furnishing

- [ ] contained in the international application as filed
- [x] filed together with the international application in electronic form
- [ ] furnished subsequently to this Authority for the purposes of search

2. [x] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/053133

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: 23-30
because they relate to subject matter not required to be searched by this Authority, namely:

The subject matter of claim 23-30 relates to a method for treatment of the human body by therapy, which does not require an intentional search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.