

(51) International Patent Classification:
C12Q 1/68 (2006.01)(21) International Application Number:
PCT/US2016/022004(22) International Filing Date:
11 March 2016 (11.03.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
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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, BN, BR, BW, BY, BZ, CA, CH, CL, 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, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

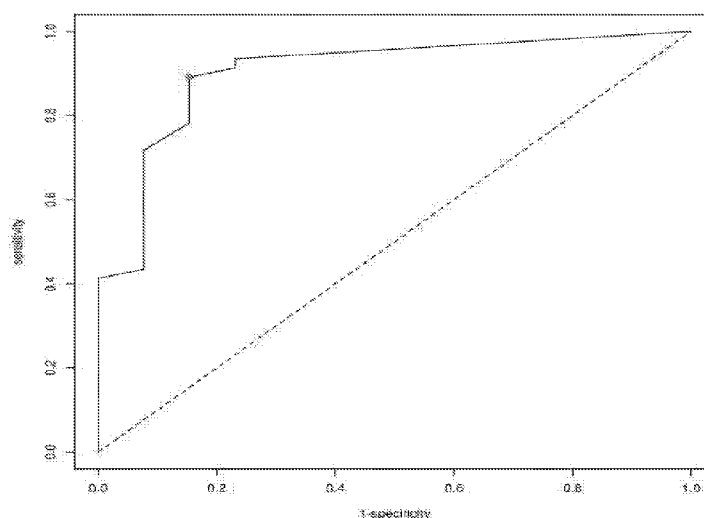
Declarations under Rule 4.17:

- 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))

[Continued on next page]

(54) Title: WHOLE BLOOD BASED MRNA MARKERS FOR PREDICTING PROSTATE CANCER AND METHODS OF DETECTING THE SAME

Figure 1



(57) Abstract: Provided are whole blood based mRNA biomarkers for predicting prostate cancer and methods of detecting the same. The disclosed mRNA markers and methods enable the identification/diagnosis of a patient with prostate cancer, identification of a patient with/prediction of high-risk prostate cancer, and treatment of a patient with prostate cancer. Also provided are gene chips comprising the disclosed whole blood based mRNA biomarkers.



Published:

- *without international search report and to be republished
upon receipt of that report (Rule 48.2(g))*

WHOLE BLOOD BASED mRNA MARKERS FOR PREDICTING PROSTATE CANCER AND METHODS OF DETECTING THE SAME

TECHNICAL FIELD

[0001] Provided herein are whole blood based mRNA biomarkers for predicting prostate cancer and methods of detecting the same. In particular, the disclosed mRNA markers and methods enable the detection of prostate cancer-specific mRNA biomarkers in a whole blood sample from a patient, identification of a patient with prostate cancer, identification of a patient with high-risk prostate cancer, and treatment of a patient with prostate cancer.

BACKGROUND

[0002] Prostate cancer is the second most common cancer among men in the United States. It is also one of the leading causes of cancer death among men of all races and Hispanic origin populations. In 2010, 196,038 men in the United States were diagnosed with prostate cancer while 28,560 men in the United States died from prostate cancer. (U.S. Cancer Statistics Working Group. United States Cancer Statistics: 1999–2010 Incidence and Mortality Web-based Report. Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute; 2013.)

[0003] One of the major challenges in management of prostate cancer is the lack of tests to distinguish between those patients who should be treated adequately to stop the aggressive form of the disease and those who should avoid overtreatment of the indolent form. Molecular heterogeneity of prostate cancer and difficulty in acquiring tumor tissue from patients makes individualized management of prostate cancer difficult.

SUMMARY

[0004] Disclosed herein are methods of detecting prostate cancer-specific mRNA biomarkers in a whole blood sample from a patient. The methods comprise, consist of and/or consist essentially of: isolating RNA from the whole blood sample; synthesizing cDNA from the isolated RNA; and measuring an expression level of at least one mRNA biomarker, wherein the at least one mRNA biomarker is or is selected from the group consisting essentially of KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV7(ARV3.7), ARV567, FOLH1, KLK2,

HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 and/or any combination thereof.

[0005] Methods for detecting ARv7(ARV3.7) in a whole blood sample from a patient are also disclosed. The methods comprise, consist of, and/or consist essentially of isolating RNA from the whole blood sample, synthesizing cDNA from the isolated RNA, and measuring an expression level of ARv7(ARV3.7).

[0006] Also disclosed are methods of identifying a patient with prostate cancer comprising, consisting of and/or consisting essentially of obtaining cDNA from a whole blood sample of the patient; contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1; measuring an expression level of COL1A1; and comparing the expression level of COL1A1 to a reference level of COL1A1, wherein an increase in the expression level of COL1A1 in the whole blood sample compared to the reference level is indicative of prostate cancer.

[0007] Methods of identifying a patient with high-risk prostate cancer are also provided. The methods comprise, consist of, and/or consist essentially of obtaining cDNA from a whole blood sample of the patient; contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for at least one mRNA biomarker indicative of high-risk prostate cancer, wherein the at least one mRNA biomarker comprises, consists of and/or consists essentially of a member selected from the group consisting of KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 and/or any combination thereof; measuring an expression level of the at least one mRNA biomarker; and comparing the expression level of the at least one mRNA biomarker to a reference level of the at least one mRNA biomarker, wherein an increase in the expression level of the at least one mRNA biomarker compared to the reference level indicates high-risk prostate cancer. In addition, mRNA biomarkers may be combined into a biomarker panel including multiple mRNA biomarkers. A subject would be called biomarker positive if greater than or equal to a predetermined, e.g., 3, 4, 5, 6, 7, 8, or 9, were detected. Biomarker positive status indicates high-risk prostate cancer.

[0008] Further disclosed are methods of treating a patient with prostate cancer comprising, consisting or, and/or consisting essentially of obtaining cDNA from a whole blood sample of the patient; contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1; measuring an expression level of COL1A1; comparing the expression level of COL1A1 to a reference level of COL1A1; and treating the patient for prostate cancer if the expression level of COL1A1 is increased compared to the reference level of COL1A1. This example is not meant to be limiting, for example expression of mRNA biomarkers or biomarker positive status described herein may also be used to select patients for treatment.

[0009] Also provided are gene chips for detecting prostate cancer specific mRNA transcripts in a whole blood sample from a patient, comprising, consisting of, and/or consisting essentially of a primer pair and a probe configured to amplify and detect a member selected from the group consisting of KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 and/or any combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The summary, as well as the following detailed description, is further understood when read in conjunction with the appended drawings. For the purpose of illustrating the disclosed methods and gene chips, there are shown in the drawings exemplary embodiments of the methods and gene chips; however, the methods and gene chips are not limited to the specific embodiments disclosed. In the drawings:

[0011] **FIG 1** represents an exemplary ROC curve for COL1A1 showing the trade-off between sensitivity and specificity at all levels of marker expression. The point indicates the optimal cutpoint which maximizes sensitivity and specificity.

[0012] **FIG 2** represents an exemplary Kaplan-Meier curve for a biomarker or biomarker panel showing the proportion of subjects that have not experienced an event such as disease recurrence or death from disease by a specific time represented on the x-axis.

Biomarker positive and biomarker negative subjects are represented by the red and black lines respectively. Biomarker positive subjects have a significantly higher likelihood of experiencing an event earlier than biomarker negative subjects, i.e. biomarker positive subjects have poorer prognosis.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0013] The disclosed methods and gene chips may be understood more readily by reference to the following detailed description taken in connection with the accompanying figures, which form a part of this disclosure. It is to be understood that the disclosed methods and gene chips are not limited to the specific methods and gene chips described and/or shown herein, and that the terminology used herein is for the purpose of describing particular embodiments by way of example only and is not intended to be limiting of the claimed methods and gene chips.

[0014] Unless specifically stated otherwise, any description as to a possible mechanism or mode of action or reason for improvement is meant to be illustrative only, and the disclosed methods are not to be constrained by the correctness or incorrectness of any such suggested mechanism or mode of action or reason for improvement.

[0015] Reference to a particular numerical value includes at least that particular value, unless the context clearly dictates otherwise. When a range of values is expressed, another embodiment includes from the one particular value and/or to the other particular value. Further, reference to values stated in ranges include each and every value within that range. All ranges are inclusive and combinable.

[0016] When values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment.

[0017] It is to be appreciated that certain features of the disclosed methods and gene chips which are, for clarity, described herein in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the disclosed methods and gene chips that are, for brevity, described in the context of a single embodiment, may also be provided separately or in any subcombination.

[0018] As used herein, the singular forms “a,” “an,” and “the” include the plural.

[0019] As used herein, the term “patient” refers to any mammal whose whole blood samples can be analyzed with the disclosed methods. Thus, the disclosed methods are applicable to human and nonhuman subjects, although it is most preferably used for humans. In some embodiments, the patient sample is a human sample. In other embodiments, the patient sample is a nonhuman sample. “Patient” and “subject” may be used interchangeably herein.

[0020] As used herein, the phrase “contacting cDNA with a gene chip” refers to a procedure whereby cDNA obtained from a whole blood sample of the patient is incubated with, or added to, a gene chip in order to evaluate gene expression.

[0021] The phrase “increase in the expression level” encompasses both the presence of the mRNA biomarker and an elevated level of the mRNA biomarker relative to a reference sample. For example, one or more of the disclosed mRNA biomarkers may be absent from a reference sample. For such mRNA biomarkers, the term “increase in the expression level” refers to the presence of the mRNA biomarker in the patient’s whole blood sample. Conversely, one or more of the disclosed mRNA biomarkers may be present at some level in a reference sample. For such mRNA biomarkers, the term “increase in the expression level” refers to an elevated level of the mRNA biomarker in the patient’s whole blood sample compared to the reference sample.

[0022] As used herein, “reference sample” refers to a whole blood sample from an individual or population of individuals that does not have, and did not in the past have, prostate cancer. Accordingly, “reference level of” refers to the level of expression of the one or more disclosed biomarkers in a whole blood sample from an individual or population of individuals that does not have, and did not in the past have, prostate cancer.

[0023] As used herein, “a primer pair” refers to a forward primer and a reverse primer for amplifying the cDNA of the mRNA biomarker of interest.

Detection of prostate cancer specific mRNA biomarkers

[0024] Disclosed herein are methods of detecting prostate cancer specific mRNA biomarkers in a whole blood sample from a patient. The methods comprise: isolating RNA from the whole blood sample; synthesizing cDNA from the isolated RNA; optionally, preamplifying the cDNA, and measuring an expression level of at least one mRNA biomarker, wherein the at least one mRNA biomarker is KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV7(ARV3.7) (also known as ARV7), ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1,

STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3, or any combination thereof.

[0025] The disclosed methods enable the detection of prostate cancer specific mRNA biomarkers in a whole blood sample. Detection of these markers can be used for identifying/diagnosing a patient with prostate cancer, identifying a patient with/predicting high-risk prostate cancer, and treating a patient with prostate cancer.

[0026] As used herein, the phrase “prostate cancer specific mRNA biomarker” refers to single mRNA biomarkers or mRNA biomarker groups (i.e., two or more associated biomarkers) which may be used to detect prostate cancer from a whole blood sample. Exemplary mRNA biomarkers are listed in Table 1 and include, but are not limited to, KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 and CYP3A5.

Table 1 – Exemplary mRNA biomarkers

mRNA Biomarker	Name	GenBank Accession No.
KLK3	kallikrein-3	NM_001030047
ACADL	Acyl-CoA Dehydrogenase, Long Chain	NM_001608.3
GRHL2	grainyhead-like 2 (Drosophila)	NM_024915.3
HOXB13	homeobox B13	NM_006361.5
HSD3B1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	NM_000862.2
TMP.ERG	TMP-ERG fusion (as described in Yu, J., <i>et al.</i> An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. <i>Cancer Cell</i> (2010) 17(5): 443-454.	
ARV3.7	Androgen receptor splice variant (as described in Hu, R., <i>et al.</i> Ligand-independent androgen receptor variants derived from splicing of cryptic exons	

	signify hormone refractory prostate cancer. Cancer Res. (2009) 69(1): 16-22.	
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	NM_001193471.1
KLK2	kallikrein-related peptidase 2	NM_001002231.2
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	NM_000198.3
AGR2	anterior gradient 2	NM_006408.3
AZGP1	alpha-2-glycoprotein 1, zinc-binding pseudogene 1	NR_036679.1
STEAP2	STEAP family member 2, metalloredutase	NM_001040665.1
KCNN2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	NM_001278204.1
GPX8	glutathione peroxidase 8 (putative)	NM_001008397.2
SLCO1B3	solute carrier organic anion transporter family, member 1B3	NM_019844.3
TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	NM_016192.2
SPINK1	serine peptidase inhibitor, Kazal type 1	NM_003122.3
SFRP4	secreted frizzled-related protein 4	NM_003014.3
NROB1	nuclear receptor subfamily 0, group B, member 1	NM_000475.4
FAM13C	family with sequence similarity 13, member C	NM_001001971.2
HNF1A	HNF1 homeobox A	NM_000545.5
CDH12	cadherin 12, type 2 (N-cadherin 2)	NM_004061.3
PGR	progesterone receptor	NM_000926.4
PITX2	paired-like homeodomain 2	NM_000325.5
MYBPC1	myosin binding protein C, slow type	NM_001254718.1
FOXA1	forkhead box A1	NM_004496.3
SRD5A2	steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)	NM_000348.3
COL1A1	collagen, type I, alpha 1	NM_000088.3
NPY	neuropeptide Y	NM_000905.3
UGT2B17	UDP glucuronosyltransferase 2 family, polypeptide B17	NM_001077.3
CLUL1	clusterin-like 1 (retinal)	NM_014410.4
C9orf152	chromosome 9 open reading frame 152	NM_001012993.2
FLNC	filamin C, gamma	NM_001127487.1
GPR39	G protein-coupled receptor 39	NM_001508.2
RELN	Reelin	NM_005045.3
THBS2	thrombospondin 2	NM_003247.2

CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	NM_000102.3
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	NM_000777.3

[0027] Suitable mRNA biomarkers can be functionally classified as androgen controlled group, abiraterone resistance group, neuroendocrine bypass group, or other bypass pathway. Thus, in some embodiments, the at least one mRNA biomarker can be a member of the androgen controlled group, abiraterone resistance group, neuroendocrine bypass group, other bypass pathway, or any combination thereof.

[0028] Expression profiling of whole blood offers several practical advantages over that of tumor tissue, including the minimally invasive nature of sample acquisition, relative ease of standardization of sampling protocols, and the ability to obtain repeated samples over time since tumor tissues are not taken as a part of standard of care. A whole blood sample can be obtained from a patient by a number of techniques known in the art including, but not limited to, venipuncture. The whole blood sample can be collected in a blood collection tube. In some embodiments, the blood collection tube can be a PAXgene® brand blood RNA tube.

[0029] Techniques for isolating RNA from a whole blood sample are known in the art. Suitable commercially available kits include, for example, QIAGEN PAXgene Blood RNA Kit, the procedure of which is described in the examples section.

[0030] Techniques for synthesizing cDNA from isolated RNA are known in the art including, but are not limited to, reverse transcription of RNA.

[0031] In some embodiments, the cDNA can be pre-amplified after the synthesizing step. The preamplifying step can be performed for any suitable number of cycles. The number of cycles depends, in part, on the amount of starting cDNA and/or the amount of cDNA required to perform the amplifying step. Suitable numbers of preamplification cycles include, but are not limited to, 1 to 20 cycles. Accordingly, the preamplification step can be performed for any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 cycles. In some aspects, the preamplification step can be performed for 2 cycles. In other aspects, the preamplification step can be performed for 14 cycles. In yet other aspects, the preamplification step can be performed for more than 20 cycles.

[0032] Measuring an expression level of at least one mRNA biomarker comprises amplifying the cDNA and detecting the amplified cDNA using a gene chip, wherein the gene chip comprises a primer pair and a probe for KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 or any combination thereof. In some aspects the gene chip can comprise a primer pair and one probe for each mRNA biomarker to be analyzed. In other aspects, the gene chip can comprise more than one primer pair and more than one probe for each mRNA biomarker to be analyzed.

[0033] Without intending to be bound by theory, the preamplified cDNA added to the gene chip will be bound and amplified by the primer pair specific for a region within the mRNA biomarker of interest. As the cDNA is amplified, the amplified cDNA will be bound by a probe specific for a region within the mRNA biomarker of interest. Binding of the probe to the amplified cDNA will enable the detection of the amplified cDNA as the amplification process is occurring. Thus, the disclosed methods enable the detection of an expression level of the at least one mRNA biomarker at an early stage in the amplification process, allowing for enhanced sensitivity and accuracy.

[0034] The measuring step can be performed on pre-amplified or non-preamplified cDNA. Amplifying cDNA can be performed, for example, by qRT-PCR. Suitable reagents and conditions are known to those skilled in the art. qRT-PCR can be performed on a number of suitable platforms. In some aspects, for example, the qRT-PCR can be performed using FluidigmTM. Accordingly, in some embodiments, the measuring step can comprise amplifying cDNA by qRT-PCR using FluidigmTM Gene expression chip on a FluidigmTM platform, wherein the gene expression chip comprises at least one mRNA biomarker as discussed above.

[0035] Also disclosed are methods for detecting ARV7(ARV3.7) in a whole blood sample from a patient. The disclosed methods comprise isolating RNA from the whole blood sample, synthesizing cDNA from the isolated RNA, and measuring an expression level of ARV3.7.

[0036] In some embodiments, the cDNA can be preamplified after the synthesizing step. Suitable numbers of preamplification cycles include, but are not limited to, 1 to 20 cycles. Accordingly, the preamplification step can be performed for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 cycles. In some aspects, the preamplification step can be performed for 2 cycles.

[0037] The whole blood sample can be collected in a blood collection tube, such as a PAXgene® blood RNA tube.

[0038] The measuring step can be performed using any one of the disclosed gene chips which comprise a primer and probe for ARV3.7. In some embodiments, the measuring step can be performed using a gene chip comprising a forward primer of SEQ ID NO:2 and a reverse primer of SEQ ID NO:3. In some aspects, the gene chip can further comprise a probe of SEQ ID NO:1.

[0039] The methods of detecting ARV7(ARV3.7)expression can further comprise comparing the expression level of ARV7(ARV3.7)from the patient's whole blood sample to a reference level of ARV7(ARV3.7)expression. Suitable reference levels of ARV7(ARV3.7)expression include, for example, the reference level of ARV7(ARV3.7)in a whole blood sample from an individual without prostate cancer. The comparing step can be used to determine if the expression level of ARV7(ARV3.7)from the patient's whole blood sample is increased or decreased relative to the reference level of ARV7(ARV3.7)expression.

Methods of identifying a patient with prostate cancer

[0040] Also disclosed herein are methods of identifying a patient with prostate cancer. The disclosed methods comprise: obtaining cDNA from a whole blood sample of the patient; contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1; measuring an expression level of COL1A1; and comparing the expression level of COL1A1 to a reference level of COL1A1, wherein an increase in the expression level of COL1A1 in the whole blood sample compared to the reference level is indicative of prostate cancer.

[0041] cDNA can be obtained from a whole blood sample by isolating RNA from the whole blood sample and synthesizing cDNA from the isolated RNA. Suitable techniques for isolating RNA and synthesizing cDNA include those disclosed above and further disclosed in the

examples section. In some embodiments, the cDNA can be pre-amplified after the synthesizing step. The preamplifying step can be performed for any suitable number of cycles including, but are not limited to, 1 to 20 cycles. Accordingly, the preamplification step can be performed for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 cycles. In some aspects, the preamplifying step can be performed for 14 cycles.

[0042] In some embodiments, the gene chip further comprises a primer pair and a probe for at least one additional mRNA biomarker, wherein the at least one additional mRNA biomarker is KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 or any combination thereof. In embodiments wherein the gene chip comprises a primer pair and a probe for at least one additional mRNA biomarker, the methods further comprises: measuring an expression level of the at least one additional mRNA biomarker; and comparing the expression level of the at least one additional mRNA biomarker to a reference level of the at least one additional mRNA biomarker, wherein an increase in the expression level of the at least one additional mRNA biomarker compared to the reference level indicates prostate cancer.

[0043] In some aspects the gene chip can comprise one primer pair and one probe for each mRNA biomarker to be analyzed. In other aspects, the gene chip can comprise more than one primer pair and more than one probe for each mRNA biomarker to be analyzed.

[0044] In embodiments wherein the gene chip further comprises a primer pair and a probe for at least one additional mRNA biomarker, the methods comprise measuring an expression level of COL1A1 and the at least one additional mRNA biomarker, and comparing the expression level of COL1A1 and the at least one additional mRNA biomarker to a reference level of COL1A1 and the at least one additional mRNA biomarker. For example, and without intending to be limiting, in some embodiments the methods can comprise contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1 and a primer pair and a probe for MYBPC1, measuring an expression level of COL1A1 and MYBPC1, and comparing the expression level of COL1A1 and MYBPC1 to a reference level of COL1A1

and MYBPC1, wherein an increase in the expression level of COL1A1 and MYBPC1 in the whole blood sample compared to the reference level is indicative of prostate cancer.

[0045] Measuring an expression level of COL1A1 alone or in combination with at least one additional mRNA biomarker comprises amplifying the cDNA with a primer pair for COL1A1 alone or in combination with a primer for at least one additional mRNA biomarker and detecting the amplified cDNA with a probe for COL1A1 alone or in combination with a probe for at least one additional mRNA biomarker. The measuring step can be performed on pre-amplified or non-preamplified cDNA. Amplifying cDNA can be performed, for example, by qRT-PCR. Suitable reagents and conditions are known to those skilled in the art. qRT-PCR can be performed on a number of suitable platforms. In some aspects, for example, the qRT-PCR can be performed using Fluidigm™. Accordingly, in some embodiments, the measuring step can comprise amplifying cDNA by qRT-PCR using Fluidigm™ Gene expression chip on a Fluidigm™ platform, wherein the gene expression chip comprises a primer for COL1A1 alone or in combination with a primer for at least one additional mRNA biomarker as discussed above.

[0046] The whole blood sample can be collected in a blood collection tube including, but not limited to, a PAX gene RNA tube.

[0047] The methods of identifying a patient with prostate cancer can further comprise confirming the expression level of the at least one mRNA biomarker by real-time PCR.

[0048] In some embodiments, the methods can further comprise assigning a risk factor to the prostate cancer, wherein an increased expression level of KLK3, PGR, KCNN2, MYBPC1, HOXB13, or any combination thereof indicates high-risk prostate cancer.

Methods of identifying a patient with high-risk prostate cancer

[0049] Methods of identifying a patient with high-risk prostate cancer are also disclosed. The methods comprise: obtaining cDNA from a whole blood sample of the patient; contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a panel of mRNA biomarkers indicative of high-risk prostate cancer, wherein the panel comprises KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 or any combination thereof; measuring an expression level of the at least one mRNA biomarker; and comparing the expression level of the at least one mRNA biomarker to a reference level of the at least one

mRNA biomarker, wherein an increase in the expression level of the at least one mRNA biomarker compared to the reference level indicates high-risk prostate cancer.

[0050] The disclosed methods enable the prediction of high risk prostate cancer based on the detection of KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 or any combination thereof from a whole blood sample.

[0051] cDNA can be obtained from a whole blood sample by isolating RNA from the whole blood sample and synthesizing cDNA from the isolated RNA. Suitable techniques for isolating RNA and synthesizing cDNA include those disclosed above and further disclosed in the examples section. In some embodiments, the cDNA can be pre-amplified after the synthesizing step. The preamplifying step can be performed for any suitable number of cycles. The number of cycles depends, in part, on the amount of starting cDNA and/or the amount of cDNA required to perform the amplifying step. Suitable numbers of preamplification cycles include, but are not limited to, 1 to 20 cycles. Accordingly, the preamplification step can be performed for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 cycles. In some aspects, the preamplification step can be performed for 2 cycles. In other aspects, the preamplification step can be performed for 14 cycles. In yet other aspects, the preamplification step can be performed for more than 20 cycles.

[0052] Measuring an expression level of KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 or any combination thereof comprises amplifying the cDNA with a primer pair for KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 or any combination thereof, and detecting the amplified cDNA with a probe for KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 or any combination thereof. The measuring step can be performed on pre-amplified or non-preamplified cDNA. Amplifying cDNA can be performed, for example, by qRT-PCR. Suitable reagents and conditions are known to those skilled in the art. qRT-PCR can be performed on a number of suitable platforms. In some aspects, for example, the qRT-PCR can be performed using Fluidigm™. Accordingly, in some embodiments, the measuring step can comprise amplifying cDNA by qRT-PCR using

Fluidigm™ Gene expression chip on a Fluidigm™ platform, wherein the gene expression chip comprises a primer for KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 or any combination thereof as discussed above.

[0053] In some aspects the gene chip can comprise one primer pair and one probe for each mRNA biomarker to be analyzed. In other aspects, the gene chip can comprise more than one primer pair and more than one probe for each mRNA biomarker to be analyzed.

[0054] The whole blood sample can be collected in a blood collection tube including, but not limited to, a PAX gene RNA tube.

[0055] The methods of identifying a patient with high-risk prostate cancer can further comprise confirming the expression level of the at least one mRNA biomarker by real-time PCR.

[0056] In some embodiments, the method can entail measuring the expression level of more than one mRNA biomarker by real-time PCR and detecting greater than a threshold number of markers with positive expression. In these embodiments, patients with greater than a threshold number of markers with positive expression are deemed to be biomarker positive. Biomarker positive status is associated with increased likelihood of high risk disease.

Methods of treating a patient with prostate cancer

[0057] Disclosed herein are methods of treating a patient with prostate cancer comprising: obtaining cDNA from a whole blood sample of the patient; contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1; measuring an expression level of COL1A1; comparing the expression level of COL1A1 to a reference level of COL1A1; and treating the patient if the expression level of COL1A1 is increased compared to the reference level of COL1A1.

[0058] In some embodiments, the gene chip further comprises a primer pair and a probe for at least one additional mRNA biomarker, wherein the at least one additional mRNA biomarker is KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 or any combination

thereof. In embodiments wherein the gene chip comprises a primer pair and a probe for at least one additional mRNA biomarker, the methods further comprise: measuring an expression level of the at least one additional mRNA biomarker; comparing the expression level of the at least one additional mRNA biomarker to a reference level of the at least one additional mRNA biomarker; and treating the patient if the expression level of the at least one additional mRNA biomarker is increased compared to the reference of the at least one additional mRNA biomarker.

[0059] In other embodiments, the gene chip further comprises a primer pair and probe for multiple mRNA biomarkers.

[0060] In some aspects the gene chip can comprise one primer pair and one probe for each mRNA biomarker to be analyzed. In other aspects, the gene chip can comprise more than one primer pair and more than one probe for each mRNA biomarker to be analyzed.

[0061] In embodiments wherein the gene chip further comprises a primer pair and a probe for at least one additional mRNA biomarker, the methods of treating a patient with prostate cancer comprise measuring an expression level of COL1A1 and the at least one additional mRNA biomarker, comparing the expression level of COL1A1 and the at least one additional mRNA biomarker to a reference level of COL1A1 and the at least one additional mRNA biomarker, and treating the patient if the expression level of COL1A1 and the at least one additional mRNA biomarker is increased compared to the reference level of COL1A1 and the at least one additional mRNA biomarker. For example, and without intending to be limiting, in some embodiments the methods can comprise contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1 and a primer pair and a probe for MYBPC1, measuring an expression level of COL1A1 and MYBPC1, comparing the expression level of COL1A1 and MYBPC1 to a reference level of COL1A1 and MYBPC1, and treating the patient if the expression level of COL1A1 and MYBPC1 is increased compared to the reference level of COL1A1 and MYBPC1.

[0062] In embodiments wherein the gene chip further comprises a primer and a probe for multiple mRNA biomarkers, the methods of treating a patient with prostate cancer comprise measuring an expression level of multiple mRNA biomarkers, comparing the expression level of mRNA biomarkers with a reference level of expression of each mRNA biomarker, and treating

the patient if greater than a threshold number of mRNA biomarkers are detected with an expression level greater than the reference level.

[0063] cDNA can be obtained from a whole blood sample by isolating RNA from the whole blood sample and synthesizing cDNA from the isolated RNA. Suitable techniques for isolating RNA and synthesizing cDNA include those disclosed above and further disclosed in the examples section. In some embodiments, the cDNA can be pre-amplified after the synthesizing step. The preamplifying step can be performed for any suitable number of cycles. The number of cycles depends, in part, on the amount of starting cDNA and/or the amount of cDNA required to perform the amplifying step. Suitable numbers of preamplification cycles include, but are not limited to, 1 to 20 cycles. Accordingly, the preamplification step can be performed for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 cycles. In some aspects, the preamplification step can be performed for 2 cycles. In other aspects, the preamplification step can be performed for 14 cycles. In yet other aspects, the preamplification step can be performed for more than 20 cycles.

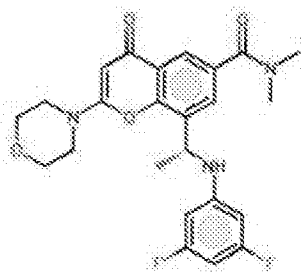
[0064] Measuring an expression level of COL1A1 alone or in combination with at least one additional mRNA biomarker comprises amplifying the cDNA with a primer pair for COL1A1 alone or in combination with a primer pair for at least one additional mRNA biomarker, and detecting the amplified cDNA with a probe for COL1A1 alone or in combination with a probe for at least one additional mRNA biomarker. The measuring step can be performed on pre-amplified or non-preamplified cDNA. Amplifying cDNA can be performed, for example, by qRT-PCR. Suitable reagents and conditions are known to those skilled in the art. qRT-PCR can be performed on a number of suitable platforms. In some aspects, for example, the qRT-PCR can be performed using Fluidigm™. Accordingly, in some embodiments, the measuring step can comprise amplifying cDNA by qRT-PCR using Fluidigm™ Gene expression chip on a Fluidigm™ platform, wherein the gene expression chip comprises a primer for COL1A1 alone or in combination with a primer for at least one additional mRNA biomarker as discussed above.

[0065] The whole blood sample can be collected in a blood collection tube including, but not limited to, a PAX gene RNA tube.

[0066] The methods of treating a patient with prostate cancer can further comprise confirming the expression level of the at least one mRNA biomarker by real-time PCR.

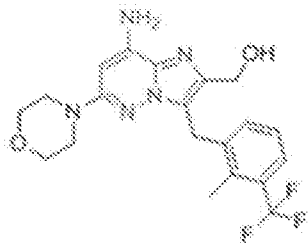
[0067] Suitable compounds for treating a patient having an increased expression level of COL1A1 or COL1A1 and the at least one additional mRNA biomarker include, but are not limited to:

[0068]



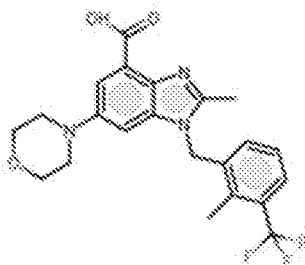
(Compound I)

[0069]



(Compound II)

[0070]



(Compound III)

Gene chips

[0071] Disclosed herein are gene chips for detecting prostate cancer specific mRNA transcripts in a whole blood sample from a patient. In some embodiments, the gene chips can comprise a primer pair and a probe configured to amplify and detect KLK3, ACADL, GRHL2,

HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3. SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 or any combination thereof.

[0072] In some embodiments, the gene chips can comprise a plurality of primer pairs and a plurality of probes configured to amplify and detect KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3. SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3 or any combination thereof. For example, and without intending to be limiting, in some aspects the gene chip can comprise one primer pair and one probe for each mRNA biomarker to be analyzed. In other aspects, the gene chip can comprise more than one primer pair and more than one probe for each mRNA biomarker to be analyzed.

[0073] Suitable probes include, but are not limited to, those listed in Table 2.

Table 2 – Exemplary probes for the disclosed gene chips

Assay ID (Life Technologies)	Target
Hs02576345_m1	KLK3
Hs00380670_m1	GPX8
Hs00165843_m1	SRD5A2
Hs01085277_m1	ACADL
Hs00227745_m1	GRHL2
H00426435_m1	HSD3B1
Hs00197189_m1	HOXB13
Hs00428383_m1	KLK2
Hs03043658_m1	NROB1
Hs00167041_m1	HNF1A
Hs01591157_m1	AZGP1P1
Hs00189528_m1	FOLH1 (PSMA)
Hs04234069_mH	PITX2(PITX2)
Hs00300346_m1	FAM13C
Hs00164004_m1	COL1A1

Hs01556702_m1	PGR
Hs00362037_m1	N-Cadherin(CDH12)
Hs00605123_m1	HSD3B2
Hs01030641_m1	KCNN2
Hs00401292_m1	STEAP2
Hs00180066_m1	SFRP4
Hs00356521_m1	AGR2
Hs00162154_m1	SPINK1
Hs00270129_m1	FOXA1
Hs00251986_m1	SLCO1B3
Hs01086906_m1	TMEFF2
Hs00159451_m1	MYBPC1
Hs00179951_m1	(Bombesin)BRS3
Hs00900370_m1	CHGA
Hs00969422_m1	LGR5
Hs00950344_m1	SNAI2

TaqMan® Reporter = FAM

[0074] Suitable primers and probes for TMP:ERG and ARV7(ARV3.7)include, but are not limited to, those listed in Table 3.

Table 3 – Exemplary primers and probes for the disclosed gene chips

Assay ID	Target	Probe Sequences	Forward Primer	Reverse Primer 5'-3'
Androgen Receptor Assays				
Custom	AR Variant 3/Variant 7 (ARV3.7)	CTGGGAGAAAA ATTCCGGGT (SEQ ID NO:1)	GGAAATGTTATGA AGCAGGGATG (SEQ ID NO:2)	TTTGAGATGCTTG CAATTGCC (SEQ ID NO:3)
Custom	AR Variant 567 (ARV567)	CTTGCCTGATTG CGAGAG (SEQ ID NO:4)	CTGGGAGAGAGAC AGCTTGTACAC (SEQ ID NO:5)	CAGGTCAAAAGTG AACTGATGCA (SEQ ID NO:6)
TMPRSS2:ETS Fusion Assays				
Custom	TMPRSS2: ERG (TMP:ERG)	CGGCAGGAAGCC TTAT (SEQ ID NO:7)	GAGCTAAGCAGGA GGCGGA (SEQ ID NO:8)	TAGGCACACTCAA ACAACGACTG (SEQ ID NO:9)
Control Gene Assays				
Custom	RPL19	CCACAAGCTGAA GGC (SEQ ID NO:10)	GCGGATTCTCATGG AACACA (SEQ ID NO:11)	GGTCAGCCAGGAG CTTCTTG (SEQ ID NO:12)

All assays used FAM TaqMan Reporter.

The control gene assays used endogenous control.

EXAMPLES

METHODS

PAXgene® blood RNA manual extraction procedure

[0075] RNA was extracted using QIAGEN's PAXgene® Blood RNA Kit as described in the PAXgene Blood RNA Kit Handbook (PreAnalytiX GmbH; March 2009), described briefly below.

[0076] The PAXgene® Blood RNA Tube was centrifuged for 10 min at 3000–5000 x g using a swing-out rotor. The supernatant was removed by decanting or pipetting. 4 ml RNase-free water was added to the pellet, and the tube was closed using a fresh secondary BD Hemogard closure. The tube was vortexed until the pellet was visibly dissolved, and centrifuged for 10 min at 3000–5000 x g. The entire supernatant was removed and discarded.

[0077] 350 µl of Buffer BR1 was added and vortexed until the pellet was visibly dissolved. The sample was pipetted into a 1.5 ml microcentrifuge tube. 300 µl Buffer BR2 and 40 µl proteinase K was added. The sample was mixed by vortexing for 5 seconds, and incubated for 10 minutes at 55°C using a shaker-incubator at 400–1400 rpm.

[0078] The lysate was pipetted directly into a PAXgene® Shredder spin column (lilac) placed in a 2 ml processing tube, and centrifuged for 3 minutes at maximum speed (but not to exceed 20,000 x g). The entire supernatant of the flow-through fraction was carefully transferred to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube. 350 µl ethanol (96–100%) was added, mixed by vortexing, and centrifuged briefly to remove drops from the inside of the tube lid.

[0079] 700 µl of sample was pipetted into the PAXgene® RNA spin column (red) placed in a 2 ml processing tube, and centrifuged for 1 min at 8000–20,000 x g. The spin column was placed in a new 2 ml processing tube. The remaining sample was pipetted into the PAXgene® RNA spin column, and centrifuged for 1 min at 8000–20,000 x g. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.

[0080] 350 µl of Buffer BR3 was pipetted into the PAXgene® RNA spin column and centrifuged for 1 min at 8000–20,000 x g. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.

[0081] 10 µl DNase I stock solution was added to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube and mixed gently. The DNase I incubation mix (80 µl) was pipetted directly onto the PAXgene® RNA spin column membrane, and placed on the bench-top (20 - 30°C) for 15 min. 350 µl of Buffer BR3 was pipetted into the PAXgene® RNA spin column, and centrifuged for 1 minute at 8,000 - 20,000 x g. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.

[0082] Another 500 µl of Buffer BR4 was added to the PAXgene RNA spin column and centrifuged for 3 minutes at 8000 - 20,000 x g. The tube containing the flow-through was discarded and the PAXgene® RNA spin column was placed in a new 2 ml processing tube and centrifuged for 1 minute at 8000 - 20,000 x g.

[0083] The tube containing the flow-through was discarded. The PAXgene® RNA spin column was placed in a 1.5 ml microcentrifuge tube, and 40 µl of Buffer BR5 was pipetted directly onto the PAXgene® RNA spin column membrane. The column was centrifuged for 1 minute at 8000–20,000 x g to elute the RNA. This step was repeated using 40 µl of Buffer BR5 and the same microcentrifuge tube.

[0084] The eluate was incubated for 5 min at 65°C in the shaker–incubator without shaking. After the incubation, the tube was chilled immediately on ice. If the RNA samples were not used immediately, they were stored at -20°C or -70°C.

cDNA Synthesis

[0085] Preparation of 2x reverse transcription master mix: Master Mix (2x) was prepared on ice as shown in Table 4 for the appropriate number of reactions (# reactions + 10%, per 20-µL reaction). Note: 10 µL of sample RNA was added to the master mix to have final reaction volume of 20 µL.

Table 4

Component	Volume (µL) for One Reaction
10X RT Buffer Mix	2
25X dNTP Mix	0.8
10X RT Random Primers	2
50U/µL MultiScribe Reverse Transcriptase	1

RNase inhibitor	1
Nuclease/RNase free H ₂ O	3.2
Total volume	10

[0086] The Master Mix was vortexed several times (5 to 10) to mix, and then centrifuged briefly (1500 x g, 5 to 10 sec). 10 µl of reaction mix was added to each appropriate well of a 96-well plate.

[0087] Addition of RNA sample to master mix: 10 µL of each RNA sample was added to the appropriate well of the 96-well plate, including the water negative control, to have a final reaction volume of 20 µL. The solutions were mixed gently by pipetting up and down 3 times. The 96-well reaction plate was sealed with a plate seal and centrifuged briefly (1500 x g for 60 seconds).

[0088] ABI 9700 set up: The ABI 9700 was set up as follows:

Step 1: 25°C for 10 min

Step 2: 37°C for 120 min

Step 3: 85°C for 5 sec

Step 4: 4°C infinite hold

Reaction volume was set to 20 µL.

cDNA preamplification

[0089] Preparation of primer probe assay mix for preamplification and real-time PCR: 100 µl of 20X Primer-Probe mixture was prepared for Real Time PCR.

[0090] Preparation of 0.2X preamp assay pool: 0.2X preamp assay pool was prepared as shown in Table 5. Note: The following volumes are for the preparation of 400 µl of 0.2X preamp assay pool. Volumes can be adjusted accordingly depending on the number of samples/Taqman assays being tested.

Table 5

Preamp Stock						
TaqMan	# of	Stock	Primer	1x TE	Final	Final

Assay	TaqMan Assays	aliquot	pool	(μ l)	Volume (μ l)	Concentration
20x concentration	48	4	192	208	400	0.2x

[0091] Sample and 2x master mix preparation: The synthesized cDNA reaction plate and 0.2X assay mix pool were placed on ice. 2X preamp master mix was prepared as shown in Table 6.

Table 6

Component	Volume (μ L) for One Reaction
2X TaqMan PreAmp Master Mix	7.5
0.2X Assay Pool 1	3.75
Total volume	11.25

[0092] 11.25 μ L of Master Mix was aliquoted into the appropriate wells of a 96-well reaction plate. 3.75 μ L of each cDNA sample, including positive and negative controls, were transferred into the appropriate wells in the Master Mix reaction plate, mixed by pipetting up and down 3 times, and centrifuging briefly.

[0093] ABI 9700 set up: The ABI 9700 was set up as follows:

Step 1: 95°C for 10 min

Step 2: 95°C for 15 sec

Step 3: 60°C for 4 min

Step 4: Set Step 2-3 for 14 cycles

Step 5: 4°C infinite hold

Reaction volume was set 15 μ L

[0094] Preamp product dilution: The PreAmp reaction plate was centrifuged briefly (1500 x g for 60 seconds) after preamplification was completed. 135 μ l of IDTE was added to each reaction well (1:10 dilution), mixed well by pipetting up and down 3 times and centrifuged briefly (1500 x g for 5 to 10 seconds). The PreAmp product was stored at -20°C until further use.

Fluidigm™ 96.96 Real-Time PCR

[0095] Preparing 10X assays: Aliquots of 10X assays were prepared using volumes in Table 7. The volumes can be scaled up appropriately for multiple runs.

Table 7

Component	Volume per Inlet (μL)	Volume per Inlet with Overage (μL)
20X TaqMan assay	2.5	3
2X Assay Loading Reagent	2.5	3
Total Volume	5.0	6

[0096] Preparing sample pre-mix and samples: The components in Table 8 were combined to make Sample Pre-Mix and final Sample Mixture. The volumes can be scaled up appropriately for multiple runs.

Table 8

Component	Volume per Inlet (μL)	Volume per Inlet with Overage (μL)
TaqMan® Genotyping Mix (2X)	2.5	3
20X GE Sample Loading Reagent	0.25	0.3
Diluted preamp	2.25	2.7
Total Volume	5.0	6

The TaqMan Genotyping Mix was combined with the GE Sample Loading Reagent in a 1.5 mL tube – enough volume to fill an entire chip. 3.3 μL of this Sample Pre-Mix can then be aliquoted for each sample.

[0097] Loading the Chip: Upon completion of the Prime (136x) script, the primed chip was removed from the IFC Controller HX. 5 μL of each assay and each sample was pipetted into their respective inlets on the chip. The chip was returned to the IFC Controller HX. Using the IFC Controller HX software, the Load Mix (136x) script was run to load the samples and assays into the chip. When the Load Mix (136x) script was complete, the loaded chip was removed from the IFC Controller.

[0098] Using the Data Collection Software: The chip was loaded on BioMark and instructions/run 96.96 specific protocols for gene expression assay were followed.

[0099] Analysis: Auto detector was selected for data analysis. CT <35 and present even in one of 4 replicates – the sample was considered positive for amplification.

AR Axis-Liquid Biopsy Assay development-methodology

[00100] Primer validation: Custom designs and revalidated Taqman gene expression assays were ordered from ABI (detailed list of markers and corresponding gene IDs are provided in Tables 2 and 3). A panel of 4 prostate cancer cell lines (VCaP, LNCaP, 22RV1 and PC-3) shown to express the majority of these genes were used for Assay and primer validation.

[00101] Validation on FFPET (formalin fixed paraffin embedded tissue) derived RNA: To test if these markers represent true aggressive tumors, the assays were tested on a set of 40 FFPE prostate cancer and adjacent normal tissue derived RNA samples. RNA from FFPET blocks were extracted using Qiagen's All Prep DNA/RNA FFPET Kit. RNA concentration was checked on Agilent BioAnalyzer. 350-500 ng of total RNA in 12 µl volume was reverse transcribed using Qiagen's Quantitect™ Reverse Transcription kit and protocol. Approximately 1/3rd of the cDNA was preamplified in a 25 µl reaction volume with 48 markers for 14 cycles using TaqMan PreAmp Master Mix/protocol. Pre amplified cDNA was diluted in a 1:20 ratio with 1x TE buffer. The diluted preamp product was loaded on 96.96 Gene Expression chip and run on Biomark following the user guide. ACTB and GAPDH were used as endogenous controls. The samples were tested in quadruplicates (described above – RNA extraction using Qiagen DNA-RNA FFPET Kit).

[00102] Cell lines spiked in PAXgene® blood: To test if the markers are differentially expressed in a background of whole blood cells (WBCs), VCaP cell lines were spiked in serial dilutions (10, 50, 100 and 500 cells) into PAXgene® (Quiagen, Valencia, CA) blood samples from normal donors. Total RNA was extracted using Qiagen's PAXgene® Blood RNA protocol (described above). RNA concentration was measured on Agilent Bioanalyzer system. 10 µl of RNA was used for cDNA prep using Applied Biosystems High Capacity cDNA Reverse Transcription kit/protocol. Approximately 1/3 of the cDNA was preamplified in a 15 µl reaction volume with 48 gene markers for 14 cycles using TaqMan PreAmp Master Mix/protocol (Applied Biosystems). Preamplified cDNA was further diluted to 1:10 ratio with 1xTE buffer. Following Fluidigm's BioMark user guide, the diluted preamp product was loaded on 96.96 Gene Expression chip and run on Biomark. Each sample and marker was tested in duplicate, thus resulting in 4 values for each gene/sample. ACTB, GAPDH and RPL19 were used as endogenous controls and BST1 and PTPRC were used as WBC Controls.

[00103] Testing PAXgene® derived RNA from Prostate cancer patients:

Approximately 170 markers were tested in PAXgene® RNA samples derived from 143 prostate cancer patients and 20 normal male subjects (cancer samples procured from Capital Biosciences, Cureline, and Conversant Bio). Markers that were differentially detected from aggressive versus indolent or normal donor were shortlisted.

[0104] RT-PCR assay on ViiA 7™ instrument to confirm BioMark results: Markers that were shown to be detected on Biomark™ (Fluidigm, San Francisco, CA) platform were further tested on ViiA7™ (Life Technologies, Carlsbad, CA) to validate/confirm the results. All the markers that are subsequently validated on ViiA7 will be shortlisted to make the liquid biopsy panel.

[0105] Fluidigm® BioMark™ HD System: This high-throughput real-time PCR assay was performed using the Fluidigm® BioMark™ HD System, which enables simultaneous detection of 96 analytes in 96 samples creating 9,216 data points from a single run. The BioMark™ HD platform uses microfluidic distribution of sample and assays requiring only 7 nL reactions and takes less than 3 hours to complete.

[0106] Methodology: RNA from FFPET, and PAXgene® RNA samples were Reverse Transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit followed by pre amplification of cDNA for 10/14 cycles using Applied Biosystems Taqman Pre-Amp Master Mix. The amplified cDNA was diluted and tested on Applied Biosystems's ViiA7™ or the Fluidigm® Biomark™ platform for gene expression.

Detection of mRNA markers specifically detected from whole blood from prostate cancer patients

[0107] Data consisting of mRNA marker expression levels from whole blood collected from prostate cancer patients and healthy controls was used to derive the assay parameters. In order to allow for independent validation of assays, data was split into training and validation sets in a 70%:30% ratio. Threshold Ct values were derived based on Receiver Operating Characteristic (ROC) analysis in the training data and diagnostic characteristics of the assays were evaluated using sensitivity, specificity and area under the ROC curve (AUC). Assays were independently evaluated on the validation data, using the threshold values obtained from the training data to predict which patients have prostate cancer and evaluating the assays with

sensitivity and specificity. Threshold Ct values determined for each marker are listed in Table 9. Individual ROC curves for representative markers are illustrated in **FIG. 1**.

Table 9 - Diagnostic information for a selected set of markers

Marker	Application	Cutpoint	Training			Validation	
			Sens	Spec	AUC	Sens	Spec
COL1A1	Diagnostic	30.7	0.891	0.846	0.904	0.778	0.875
MYBPC1	Diagnostic, High risk	29.4	0.783	0.769	0.746	0.722	0.375
FAM13C	Diagnostic	29.2	0.674	0.692	0.719	0.722	0.5
GPX8	Diagnostic	32.9	0.652	0.692	0.676	0.556	0.875
HOXB13	Diagnostic, High risk	31.4	0.457	0.846	0.648	0.278	1
SFRP4	Diagnostic	30.1	0.609	0.769	0.635	0.5	0.5
PITX2	Diagnostic	29.9	0.5	0.769	0.626	0.5	0.625
PGR	High risk	30.3	0.457	0.538	0.419	0.389	0.375
KLK3	High risk	39.1	0.152	1	0.576	0.167	1
KCNN2	High risk	36.4	0.652	0.462	0.492	0.667	0.75

Markers are represented by gene symbol. Cutpoint indicates the threshold Ct value of detection for each marker. Sensitivity (Sens) is equal to the probability that the marker will be detected in a patient with prostate cancer. Specificity is equal to the probability that the marker will not be detected in a patient without prostate cancer. The area under the ROC curve (AUC) is the probability that the marker will be expressed at a higher level in prostate cancer patients relative to healthy controls.

Prediction of high-risk prostate cancer based on detection of mRNA markers from whole blood

[0108] The prognostic power of selected markers was evaluated in a dataset consisting of expression levels of mRNA markers in whole blood collected from prostate cancer patients and healthy controls. Threshold values were derived using ROC analysis described above to discriminate between prostate cancer and normal healthy controls in training data. Association

with clinical risk factors was evaluated using the Fisher Exact test. Selected markers which are significantly associated with clinical risk factors are listed in Tables 10 – 12.

Table 10 - Association of presence/absence of mRNA markers with Gleason score

Marker	Expression	N	GLEASON. LOW	GLEASON.HI GH	p.val
HOXB13	Absent	38	31	7	-
	Present	26	12	14	0.0060
PGR	Absent	36	19	17	-
	Present	28	24	4	0.0072

Marker expression was dichotomized using thresholds derived for optimal detection of prostate tumors vs. normal healthy controls. Patients were dichotomized into low (Gleason ≤ 6) and high (Gleason ≥ 8) risk subsets using Gleason scores from diagnosis. The table shows the total number of tumors with/without expression of each marker (N) and the tabulation of marker detection in Gleason Low and Gleason High risk groups. Significant association is determined by the Fisher Exact Test (p.val).

Table 11 - Association of presence/absence of mRNA markers with PSA levels measured at diagnosis

Transcript	Expression	N	PSA.LOW	PSA.HIGH	p.val
KLK3	Absent	54	44	10	-
	Present	9	4	5	0.0287
PGR	Absent	35	23	12	-
	Present	28	25	3	0.0385

Marker expression was dichotomized using thresholds derived for optimal detection of prostate tumors vs. normal healthy controls. Patients were dichotomized into low (PSA < 20 ng/mL) and high (PSA ≥ 20 ng/mL) risk subsets. The table shows the total number of tumors with/without expression of each marker (N) and the tabulation of marker detection in PSA Low and PSA High risk groups. Significant association is determined by the Fisher Exact Test (p.val).

Table 12 - Association of presence/absence of mRNA markers with metastasis

Transcript	Expression	N	LOCAL	MET	p.val
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PGR	Absent	36	5	31	-
	Present	28	13	15	0.0055
KCNN2	Absent	22	2	20	-
	Present	42	16	26	0.0187
MYBPC1	Absent	15	1	14	-
	Present	49	17	32	0.0482

Marker expression was dichotomized using thresholds derived for optimal detection of prostate tumors vs. normal healthy controls. The table shows the total number of tumors with/without expression of each marker (N) and the tabulation of marker detection in tumors with/without metastatic recurrence. Significant association is determined by the Fisher Exact Test (p.val).

Identification of a multivariate biomarker panel indicative of high risk prostate cancer

A biomarker panel including multiple mRNA biomarkers was identified in a dataset consisting of expression levels of mRNA markers in whole blood collected from prostate cancer patients and healthy controls. Sixteen gene (ACADL, AGR2, COL1A1, FAM13C, GPX8, GRHL2, HNF1A, HOXB13, KLK2, KLK3, MYBPC1, NR0B1, PITX2, SFRP4, SLCO1B3, TMEFF2) were identified with significantly higher expression in prostate cancer samples relative to healthy volunteers. These 16 genes were combined into a multivariate biomarker panel as described below. Threshold values were derived using ROC analysis as described above. Thresholds were selected to identify prostate cancer patients with 90% specificity, i.e. 90% or more of healthy volunteers would be correctly identified as healthy volunteers. A machine learning process was used to define the number of detected positive biomarkers at which a patient should be deemed high risk. In order to guard against overfitting, the data was split into training and validation sets as described above. The training set is used to define the classification rule including the optimal number of positive markers. Specifically, bootstrap samples were generated by randomly selecting 100 samples from the training data. Classification rules were created by evaluating the correlation of the predicted biomarker status with the time to biochemical recurrence.

Correlation was measured using the concordance index, which measures the probability that a subject with a biomarker positive state will experience biochemical recurrence prior to a subject with a biomarker negative state. Eight markers was identified as the optimal number of features for the classification rule, i.e., subjects with greater than 8 markers positive would be deemed to

be biomarker positive and would be predicted to have shorter time to biochemical recurrence. The association between the classification rule and time to biochemical recurrence was validated using the independent validation set of samples using cox regression analysis. The multivariate biomarker panel identified a subset of subjects with significantly shorter time to biochemical recurrence compared to the biomarker negative population (HR = 7.94, p-value = 0.024).

[0109] Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments of the invention and that such changes and modifications can be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

[0110] The disclosures of each patent, patent application, and publication cited or described in this document are hereby incorporated herein by reference, in its entirety.

What is Claimed:

1. A method of detecting prostate cancer specific mRNA biomarkers in a whole blood sample from a patient comprising:
 - isolating RNA from the whole blood sample;
 - synthesizing cDNA from the isolated RNA; and
 - measuring an expression level of at least one mRNA biomarker, wherein the at least one mRNA biomarker is selected from the group consisting of KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3, and any combination thereof.
2. The method of claim 1, wherein the cDNA is preamplified after the synthesizing step.
3. The method of claim 2, wherein preamplifying step is performed for 14 cycles.
4. The method claims 2 or 3, wherein the amplifying step is performed by qRT-PCR.
5. The method of any one of the previous claims, wherein the whole blood sample is collected in a blood collection tube.
6. A method of identifying a patient with prostate cancer comprising:
 - obtaining cDNA from a whole blood sample of the patient;
 - contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1;
 - measuring an expression level of COL1A1; and
 - comparing the expression level of COL1A1 to a reference level of COL1A1, wherein an increase in the expression level of COL1A1 in the whole blood sample compared to the reference level is indicative of prostate cancer.

7. The method of claim 6, wherein the gene chip further comprises a primer pair and a probe for at least one additional mRNA biomarker, wherein the at least one additional mRNA biomarker is selected from the group consisting of KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3, and any combination thereof;
and wherein the method further comprises:
measuring an expression level of the at least one additional mRNA biomarker;
and
comparing the expression level of the at least one additional mRNA biomarker to a reference level of the at least one additional mRNA biomarker, wherein an increase in the expression level of the at least one additional mRNA biomarker compared to the reference level indicates prostate cancer.
8. The method of claim 6 or 7, wherein the cDNA is preamplified after the synthesizing step.
9. The method of claim 8, wherein preamplifying step is performed for 14 cycles.
10. The method claims 8 or 9, wherein the amplifying step is performed by qRT-PCR.
11. The method of any one of claims 6-10, wherein the whole blood sample is collected in a PAX gene RNA tube.
12. The method of any one of claims 6-11, further comprising confirming the expression level of the at least one mRNA biomarker by real-time PCR.

13. The method of any one of claims 6-12, further comprising assigning a risk factor to the prostate cancer, wherein an increased expression level of KLK3, PGR, KCNN2, MYBPC1, HOXB13, or any combination thereof indicates high-risk prostate cancer.
14. A method of identifying a patient with high-risk prostate cancer comprising:
- obtaining cDNA from a whole blood sample of the patient;
 - contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for at least one mRNA biomarker indicative of high-risk prostate cancer, wherein the at least one mRNA biomarker comprises KLK3, PGR, KCNN2, MYBPC1, HOXB13, or any combination thereof;
 - measuring an expression level of the at least one mRNA biomarker; and
 - comparing the expression level of the at least one mRNA biomarker to a reference level of the at least one mRNA biomarker, wherein an increase in the expression level of the at least one mRNA biomarker compared to the reference level indicates high-risk prostate cancer.
15. A method of treating a patient with prostate cancer comprising:
- obtaining cDNA from a whole blood sample of the patient;
 - contacting the cDNA with a gene chip, wherein the gene chip comprises a primer pair and a probe for COL1A1;
 - measuring an expression level of COL1A1;
 - comparing the expression level of COL1A1 to a reference level of COL1A1; and
 - treating the patient if the expression level of COL1A1 is increased compared to the reference level of COL1A1.
16. The method of claim 15, wherein the gene chip further comprises a primer pair and a probe for at least one additional mRNA biomarker, wherein the at least one additional mRNA biomarker is selected from the group consisting of KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1P1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, NPY,

UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3, SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3, and any combination thereof;

and wherein the method further comprises:

measuring an expression level of the at least one additional mRNA biomarker;

comparing the expression level of the at least one additional mRNA biomarker to a reference level of the at least one additional mRNA biomarker; and

treating the patient if the expression level of the at least one additional mRNA biomarker is increased compared to the reference of the at least one additional mRNA biomarker.

17. A method for detecting ARV7(ARV3.7)in a whole blood sample from a patient comprising:

isolating RNA from the whole blood sample;

synthesizing cDNA from the isolated RNA; and

measuring an expression level of ARV3.7.

18. The method of claim 17, wherein the cDNA is preamplified after the synthesizing step.

19. The method of claim 18, wherein the preamplifying step is performed for 2 to 14 cycles.

20. The method of any one of claims 17-19, wherein the whole blood sample is collected in a blood collection tube.

21. The method of any one of claims 17-20, wherein the measuring step is performed using a gene chip comprising a forward primer of SEQ ID NO:2 and a reverse primer of SEQ ID NO:3.

22. The method of claim 21, wherein the gene chip further comprises a probe of SEQ ID NO:1

23. The method of any one of claims 17-22, further comprising comparing the expression level of ARV7(ARV3.7)from the patient's whole blood sample to a reference level of ARV7(ARV3.7)expression.
24. The method of claim 23, wherein the reference level of ARV7(ARV3.7)is an expression level of ARV7(ARV3.7)in a whole blood sample from an individual without prostate cancer.
25. The method of claim 22 or 23, further comprising determining if the expression level of ARV7(ARV3.7)from the patient's whole blood sample is increased or decreased relative to the reference level of ARV7(ARV3.7)expression.
26. A gene chip for detecting prostate cancer specific mRNA transcripts in a whole blood sample from a patient, comprising a primer pair and a probe configured to amplify and detect an mRNA biomarker selected from the group consisting of KLK3, ACADL, GRHL2, HOXB13, HSD3B1, TMP.ERG, ARV3.7, ARV567, FOLH1, KLK2, HSD3B2, AGR2, AZGP1, STEAP2, KCNN2, GPX8, SLCO1B3, TMEFF2, SPINK1, SFRP4, NROB1, FAM13C, HNF1A, CDH12, PGR, PITX2, MYBPC1, FOXA1, SRD5A2, COL1A1, NPY, UGT2B17, CLUL1, C9orf152, FLNC, GPR39, RELN, THBS2, CYP17A1, CYP3A5, BRS3. SNAI2, CDH12, NKX3.1, LGR5, TRPM8, SLCO1B3, and any combination thereof.
27. A method of identifying a patient with high-risk prostate cancer comprising:
 - obtaining cDNA from a whole blood sample of the patient;
 - contacting the cDNA with a gene chip, wherein the gene chip comprises a set of primer pair and a probes for eight mRNA biomarkers indicative of high-risk prostate cancer, the at least 8 mRNA biomarkers is selected from the group consisting of KLK3, PGR, KCNN2, MYBPC1, HOXB13, COL1A1, GPX8, FAM13C, SLCO1B3, KLK2, TMEFF2, NROB1, PITX2, ACADL, SFRP4, AGR2, HNF1A, GRHL2 or any combination thereof;
 - measuring expression levels of all mRNA biomarker(s); and

comparing the expression level of the at least eight mRNA biomarkers to a reference level of the at least 8 mRNA biomarkers, wherein an increase in the expression level of the at least eight mRNA biomarkers compared to the reference level of the at least eight mRNA biomarkers indicates high-risk prostate cancer.

Figure 1

