

NOVEL MARKER FOR DETECTION OF BLADDER CANCER AND/OR INFLAMMATORY CONDITIONS OF THE BLADDER

FIELD OF THE INVENTION

This invention relates to the detection of disease. Specifically, this invention relates to the use of genetic and/or protein markers for detection of bladder disease, and more particularly to the use of genetic and/or protein markers for detection of transitional cell carcinoma of the bladder (TCC) or for the detection of inflammatory conditions of the bladder.

BACKGROUND

Introduction

Survival of cancer patients is greatly enhanced when the cancer is treated early. In the case of bladder cancer, patients diagnosed with disease that is confined to the primary site have a 5 year survival rate of 73%, compared to 6% for patients with metastatic disease (Altekruse et al). Therefore, developments that lead to early and accurate diagnosis of bladder cancer can lead to an improved prognosis for the patients. To aid in early detection of cancer a number of cancer specific markers have been identified. However the use of these markers can result in false positive results in patients having inflammatory bladder diseases, and not bladder cancer.

Several tests have received FDA approval for bladder cancer diagnosis or monitoring including NMP22[®] ELISA (a registered trademark of Matritech, Inc., of Massachusetts, United States) and point-of-care BladderChek[®] assays (a registered trademark of Matritech, Inc. of Massachusetts, United States), UroVysion[®] (a registered trademark of Abbott Laboratories, Inc., of Illinois, United States), ImmunoCyt[®] (a registered trademark of Sanofi Pasteur Limited/Sanofi Pasteur Limitee, Ontario, Canada and Aventis Pasteur Limited/Adventis Pasteur Limitee,, Ontario, Canada) and BTA (bladder tumour antigen), although none have demonstrated sufficient performance to routinely displace cytology

SUMMARY OF THE INVENTION

There is a need for further markers that are specific for inflammatory disease in bladder, both for the diagnosis of inflammatory disease in the bladder and also to improve the diagnosis of transitional cell carcinoma of the bladder (TCC). Specifically, there is a need for markers to allow the differentiation of TCC from other inflammatory conditions.

Aspects of this invention provide methods, compositions and devices that can provide for detection of inflammatory disease in the bladder and also to decrease the frequency of false positive results in the diagnosis of transitional cell carcinoma of the bladder (TCC).

Proteins or nucleic acids that are secreted by or cleaved from the cell, or lost by apoptotic mechanisms, either alone or in combination with each other, have utility as serum or body fluid markers for the diagnosis of disease, including inflammatory disease in bladder and/or bladder cancer or as markers for monitoring the progression of established disease. Detection of protein and cell markers can be carried out using methods known in the art, and include the use of RT-PCT, qRT-PCR, monoclonal antibodies, polyclonal antisera and the like.

Specifically the present invention provides for a method for detecting or diagnosing bladder cancer, comprising: (i) providing a biological sample; and (ii) detecting the levels of human neutrophil marker interleukin 8 receptor B (IL8Rb) in association with one or more bladder tumour markers (BTM) in said sample. The presence of cancer can be established by comparing the levels of IL8Rb and the one or BTM with the levels in normal patients, patients having bladder cancer, and/or patients having an inflammatory disease. For example, the presence of cancer can be established by comparing the expression of the marker, including IL8Rb, against a threshold. The threshold may be in the order of expression that is at least 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, or 10 times normal expression or higher. A high expression of IL8Rb can be indicative of an inflammatory disease rather than cancer.

The method of the present invention can be used in conjunction with any suitable marker for detecting bladder cancer. Examples of suitable markers for use in the invention are outlined in Figure 6 or Figure 7. The present invention includes the use of any one or more of the markers outlined in Figure 6 or Figure 7 in conjunction with IL8Rb to detect bladder cancer in a patient.

In particular, the present invention involves the use of IL8Rb in conjunction with any one or more of the markers MDK, CDC2, HOXA13, and IGFBP5 to diagnosis bladder cancer. That is, the present invention also includes any combination of IL8Rb with one or more of the markers MDK, CDC2, HOXA13, and IGFBP5, which can also be in combination with one or more other marker suitable for detecting bladder cancer, for example, any one of more of the markers outlined in Figure 6 or Figure 7. Specifically, the present invention includes any one

of the combination of markers: IL8Rb / MDK, IL8Rb / CDC2, IL8Rb / HOXA13, IL8Rb / IGFBP5, IL8Rb / MDK / CDC2, IL8Rb / MDK / HOXA13, IL8Rb / MDK / IGFBP5, IL8Rb / CDC2 / HOXA13, IL8Rb / CDC2 / IGFBP5, IL8Rb / HOXA13 / IGFBP5, IL8Rb / MDK / CDC2 / HOXA13, IL8Rb / MDK / CDC2 / IGFBP5, IL8Rb / CDC2 / HOXA13 / IGFBP5, and IL8Rb / MDK / CDC2 / HOXA13 / IGFBP5. These combinations can optionally include one or more further markers suitable for detecting bladder cancer, for example any one of more of the markers outlined in Figure 6 or Figure 7.

The present invention also provides for a method for detecting inflammatory conditions of the bladder, comprising: (i) providing a biological sample from a patient; and (ii) detecting the levels of human neutrophil marker interleukin 8 receptor B (IL8Rb) in said sample. The presence of inflammatory conditions of the bladder is established by comparing the levels of IL8Rb with the levels in normal patients, and patients having an inflammatory condition of the bladder. For example, the presence of an inflammatory condition of the bladder can be established by comparing the expression of the marker, including IL8Rb, against a threshold. The threshold may be in the order of expression that is at least 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, or 10 times normal expression or higher.

The methods of the present invention can be carried out by detecting any suitable marker of gene expression, for example determining the levels of mRNA, cDNA, a protein or peptide utilizing, any suitable method.

The establishment of a diagnosis can be established through the use classifier system, for example Linear Discriminant Analysis (LDA), Logistic Regression (LogReg), Support Vector Machine (SVM), K-nearest 5 neighbors (KN5N), and Partition Tree Classifier (TREE).

In another embodiment the invention provides for a device for detecting a bladder cancer, and or an inflammatory condition of the bladder, comprising: a substrate having a IL8Rb capture reagent and capture reagent for one or more bladder tumour marker (BTM) thereon; and a detector associated with said substrate, said detector capable of detecting a the expression associated with said capture reagent.

The invention also provides for a kit for detecting bladder cancer, comprising: a substrate having a IL8Rb capture reagent and a capture reagent for one or more bladder tumour markers (BTM) thereon; a means for visualizing a complex of said capture agent and a marker; reagents; and instructions for use.

In a further embodiment, the invention provides for a method for detecting bladder cancer, comprising the steps of: providing a test sample from a patient at risk of having bladder cancer; measuring the expression of IL8Rb protein and one or more bladder tumour marker (BTM) in said test sample; and comparing the amount of IL8Rb expression and one or more BTMs present in said test sample with a value obtained from one or more control samples from a subjects not having bladder cancer, and/or with one ore more control samples with subjects having an inflammatory condition of the bladder.

The present invention also provides for a method for screening for bladder cancer, comprising the steps of: providing a test sample from a test subject; measuring the presence of IL8Rb and at one or more bladder tumour markers (BTM) in said test sample; and comparing the amount of markers present in said test sample with a value obtained from one or more control samples from subjects not having bladder cancer, and/or with control samples with one or more subjects having an inflammatory condition of the bladder.

It has surprisingly been found that IL8Rb can detect the presence of and inflammatory bladder disease, and that this can be used to increase the ability of a marker for bladder cancer to accurately detect bladder cancer in a patient by reducing the potential of false positive results caused by an inflammatory bladder condition.

BRIEF DESCRIPTION OF THE FIGURES

This invention is described with reference to specific embodiments thereof and with reference to the figures, in which:

- Figure 1 shows the protein and mRNA sequences of IL8Rb (also known as CXCR2).
- Figure 2 shows scatter plots showing the effect of *IL8Rb* on the separation of TCC from non-malignant disease (cystitis, urinary tract infection and urolithiasis). *IL8Rb* has been substituted for different bladder cancer RNA markers in fig 2c and 2f. (a). *MDK/IGFBP5*; (b). *MDK/HOXA13*; (c) *MDK/IL8Rb*; (d) *CDC2/IGFBP5*; (e). *CDC2/HOXA13*; (f). *CDC2/IL8Rb*.
- Figure 3 ROC curve analysis (sensitivity vs specificity) showing the effect of including *IL8Rb* in the diagnostic algorithms derived using linear discriminate analysis (LD) and linear regression (LR). The ROC curves are derived from patients with TCC (n=56) and the non-malignant diseases cystitis, urinary tract

infection and urolithiasis (n=61). (a). LD1 (solid) and LD2 (dashed). (b) LR1 (solid) and LR2 (dashed). *IL8Rb* is included in LD2 and LR2.

- Figure 4 Extended ROC curve analysis showing the effect of including *IL8Rb* in the diagnostic algorithms derived using linear discriminate analysis (LD) and linear regression (LR). The ROC curves are derived from patients with TCC (n=56) and, unlike Fig 3, any non-malignant disease in the cohort (n=386). (a). LD1 (solid) and LD2 (dashed). (b) LR1 (dashed) and LR2 (solid). *IL8Rb* is included in LD2 and LR2.
- Figure 5 shows box plots showing the accumulation of *IL8Rb* mRNA in the urine of patients with non-malignant urological disease. The RNA has been quantified by qRT-PCR using the delta-Ct method (Holyoake et al, 2008). With this method a lower Ct reflects higher RNA levels. BPH: benign prostatic hyperplasia; UTI: urinary tract infection; NS prostate: non-specific prostate diseases; Vasc. Prostate: vascular prostate; warfarin: hematuria secondary to warfarin use. The observations in patients with cystitis/UTI are significantly different (p=0.001) to the other non-malignant presentations shown.
- Figure 6 shows markers known to be over expressed in bladder cancer, and are suitable for use in the present invention.
- Figure 7 shows markers known to be under expressed in bladder cancer, and are suitable for use in the present invention.
- Figure 8 shows a flow chart for the patient recruitment procedures and numbers for Example 2.
- Figure 9 Baseline clinical and demographic characteristics of the patients by disease status at 3 months.
- Figure 10 shows overall sensitivity and specificity of the urine tests.
- Figure 11 shows various ROC curves; 11a. ROC curves for NMP22 ELISA and uRNA-D (test comprising the four markers MDK+CDC2+IGFBP5+HOXA13); and 11b. ROC curve for the five markers MDK, CDC2, HOXA13, IGFBP5 and *IL8Rb*.

- Figure 12 shows the sensitivity of urine tests by stage, grade, location of tumour, multiplicity of tumour, haematuria status, creatinine of urine sample and sex. Tables show numbers and percent with a positive urine test among those with TCC.
- Figure 13 shows the specificity of urine tests by diagnosis, microhaematuria, creatinine and sex. Tables show number and % with a negative urine test result among those without TCC.
- Figure 14 shows ROC curves for the combinations of markers: (a) MDK, (b) CDC, (c) IGFBP5, (d) HOXA13, (e) MDK+CDC2, (f) MDK+IGFBP5, (g) MDK+HOXA13, (h) CDC2+IGFBP5, (i) CDC+HOXA13, (j) IGF+HOXA13, (k) MDK+CDC2+IGFBP5, (l) MDK+CDC2+HOXA13, (m) MDK+IGFBP5+HOXA13, (n) CDC2+IGFBP5+HOXA13, (o) MDK+CDC2+IGFBP5+HOXA13, plus or minus IL8Rb, using five different classifier models (i) Linear Discriminant Analysis (LDA), (ii) Logistic Regression (LogReg), (iii) Support Vector Machine (SVM), (iv) K-nearest 5 neighbors (KN5N), and (v) Partition Tree Classifier (TREE).
- Figure 15 shows (a) the "Area Under the Curve" (AUC) for up to 20% false positive rate (80% specificity) of the ROC curves from figure 14 and (b) shows the difference the AUC resulting from the inclusion of IL8Rb.
- Figure 16 shows the sensitivity of the combinations of the four markers MDK, CDC2, IGFBP5, and HOXA13, plus or minus IL8Rb, using five different classifier models (i) Linear Discriminant Analysis (LDA), (ii) Logistic Regression (LogReg), (iii) Support Vector Machine (SVM), (iv) K-nearest 5 neighbors (KN5N), and (v) Partition Tree Classifier (TREE), at different set specificities; (a) 80%, (b) 85%, (c) 90%, (d) 95%, (e) 98%.
- Figure 17 shows the resulting gains in sensitivity from adding IL8Rb at different set specificities (a) 80%, (b) 85%, (c) 90%, (d) 95%, (e) 98%, and the resulting gains in specificity from adding IL8Rb at different set specificities (f) 80%, (g) 85%, (h) 90%, (i) 95%, (j) 98%

DETAILED DESCRIPTION

Definitions

Before describing the embodiments of the invention in detail, it will be useful to provide some definitions of terms as used herein.

The term "marker" refers to a molecule that is associated quantitatively or qualitatively with the presence of a biological phenomenon. Examples of "markers" include a polynucleotide, such as a gene or gene fragment, RNA or RNA fragment; or a gene product, including a polypeptide such as a peptide, oligopeptide, protein, or protein fragment; or any related metabolites, by products, or any other identifying molecules, such as antibodies or antibody fragments, whether related directly or indirectly to a mechanism underlying the phenomenon. The markers of the invention include the nucleotide sequences (e.g., GenBank sequences) as disclosed herein, in particular, the full-length sequences, any coding sequences, any fragments, or any complements thereof, and any measurable marker thereof as defined above.

As used herein "antibodies" and like terms refer to immunoglobulin molecules and immunologically active portions of immunoglobulin (Ig) molecules, i.e., molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. These include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fc, Fab, Fab', and Fab₂ fragments, and a Fab expression library. Antibody molecules relate to any of the classes IgG, IgM, IgA, IgE, and IgD, which differ from one another by the nature of heavy chain present in the molecule. These include subclasses as well, such as IgG1, IgG2, and others. The light chain may be a kappa chain or a lambda chain. Reference herein to antibodies includes a reference to all classes, subclasses, and types. Also included are chimeric antibodies, for example, monoclonal antibodies or fragments thereof that are specific to more than one source, e.g., a mouse or human sequence. Further included are camelid antibodies, shark antibodies or nanobodies.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by abnormal or unregulated cell growth. Cancer and cancer pathology can be associated, for example, with metastasis, interference with the normal functioning of neighbouring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, neoplasia, premalignancy, malignancy, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc.

The term "tumour" refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

The term "bladder cancer" refers to a tumour originating in the bladder. These tumours are able to metastasize to any organ.

The term "BTM" or "bladder tumour marker" or "BTM family member" means a tumour marker (TM) that is associated with bladder cancer and transitional cell carcinoma of the bladder (TCC). The term BTM also includes combinations of individual markers, whose combination improves the sensitivity and specificity of detecting bladder cancer. It is to be understood that the term BTM does not require that the marker be specific only for bladder tumours. Rather, expression of BTM can be altered in other types of cells, diseased cells, tumours, including malignant tumours.

The term "under expressing BTM" means a marker that shows lower expression in bladder tumours than in non-malignant bladder tissue.

The term "over expressing BTM" means a marker that shows higher expression in bladder tumours than in non-malignant tissue.

The terms "differentially expressed," "differential expression," and like phrases, refer to a gene marker whose expression is activated to a higher or lower level in a subject (e.g., test sample) having a condition, specifically cancer, such as melanoma, relative to its expression in a control subject (e.g., reference sample). The terms also include markers whose expression is activated to a higher or lower level at different stages of the same condition; in diseases with a good or poor prognosis; or in cells with higher or lower levels of proliferation. A differentially expressed marker may be either activated or inhibited at the polynucleotide level or polypeptide level, or may be subject to alternative splicing to result in a different polypeptide product. Such differences may be evidenced by a change in mRNA levels, surface expression, secretion or other partitioning of a polypeptide, for example.

Differential expression may include a comparison of expression between two or more markers (e.g., genes or their gene products); or a comparison of the ratios of the expression between two or more markers (e.g., genes or their gene products); or a comparison of two differently processed products (e.g., transcripts or polypeptides) of the same marker, which

differ between normal subjects and diseased subjects; or between various stages of the same disease; or between diseases having a good or poor prognosis; or between cells with higher and lower levels of proliferation; or between normal tissue and diseased tissue, specifically cancer, or melanoma. Differential expression includes both quantitative, as well as qualitative, differences in the temporal or cellular expression pattern in a gene or its expression products among, for example, normal and diseased cells, or among cells which have undergone different disease events or disease stages, or cells with different levels of proliferation.

The term "expression" includes production of polynucleotides and polypeptides, in particular, the production of RNA (e.g., mRNA) from a gene or portion of a gene, and includes the production of a polypeptide encoded by an RNA or gene or portion of a gene, and the appearance of a detectable material associated with expression. For example, the formation of a complex, for example, from a polypeptide-polypeptide interaction, polypeptide-nucleotide interaction, or the like, is included within the scope of the term "expression". Another example is the binding of a binding ligand, such as a hybridization probe or antibody, to a gene or other polynucleotide or oligonucleotide, a polypeptide or a protein fragment, and the visualization of the binding ligand. Thus, the intensity of a spot on a microarray, on a hybridization blot such as a Northern blot, or on an immunoblot such as a Western blot, or on a bead array, or by PCR analysis, is included within the term "expression" of the underlying biological molecule.

The terms "gene expression threshold," and "defined expression threshold" are used interchangeably and refer to the level of a marker in question, outside which the expression level of the polynucleotide or polypeptide serves as a predictive marker for a condition in the patient. For example, the expression of IL8Rb above a certain threshold is diagnostic that the patient has an inflammatory condition. A threshold can also be used when testing a patient for suspected bladder cancer, using bladder cancer makers. Expression levels above a threshold indicates that the patient has an inflammatory bladder condition, likely to cause a false positive test for cancer, whereas an expression level of IL8Rb below a threshold is predictive that the patient does not have an inflammatory bladder condition. By including the measurement of IL8Rb any result from the expression of the bladder tumour markers can be relied upon if the levels of IL8Rb is below the threshold (i.e. a positive result is likely to be positive for the patient having cancer rather than increased levels of the bladder tumour markers actually resulting from exfoliation of non-malignant cells from the mucosa from inflammation).

The term "diagnostic threshold" refers to a threshold in which a patient can be said to have been diagnosed either with or without a given condition, for example bladder cancer. A diagnostic threshold is generally set to achieve a desired sensitivity and specificity, depending on factors such as population, prevalence, and likely clinical outcome. In general the diagnostic threshold can be calculated and/or established using algorithms, and/or computerized data analysis.

The exact threshold will be dependent on the population and also any model being used to predict disease (predictive model). A threshold is established experimentally from clinical studies such as those described in the Examples below. Depending on the prediction model used, the expression threshold may be set to achieve maximum sensitivity, or for maximum specificity, or for minimum error (maximum classification rate). For example a higher threshold may be set to achieve minimum errors, but this may result in a lower sensitivity. Therefore, for any given predictive model, clinical studies will be used to set an expression threshold that generally achieves the highest sensitivity while having a minimal error rate. In general the threshold is likely to be in the order of expression that is at least 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, or 10 times normal expression or higher.

The term "sensitivity" means the proportion of individuals with the disease who test (by the model) positive. Thus, increased sensitivity means fewer false negative test results.

The term "specificity" means the proportion of individuals without the disease who test (by the model) negative. Thus, increased specificity means fewer false positive test results.

The term "ROC curve", or Receiver Operating Characteristic curve means a plot of the true positive rate (sensitivity) against the false positive rate (specificity) for different cut off points for a particular marker or test. Each point on the ROC curve represents a specific sensitivity/specificity point that will correspond to a given threshold. The ROC curve can be important to establish a threshold to give a desired outcome. The area under a ROC curve represents (expressed as an AUC analysis), can be a measure of how well a given marker or test can distinguish between two diagnostic outcomes. The ROC curve can also be used to compare the accuracy of two different tests.

The term "oligonucleotide" refers to a polynucleotide, typically a probe or primer, including, without limitation, single-stranded deoxyribonucleotides, single- or double-stranded ribonucleotides, RNA: DNA hybrids, and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA probe oligonucleotides, are often synthesized by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available, or by a variety of other methods, including *in vitro* expression systems, recombinant techniques, and expression in cells and organisms.

The term "overexpression" or "overexpressed" refers to an expression level of a gene or marker in a patient that is above that seen in normal tissue. Expression may be considered to be overexpressed if it is 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, or greater than 2 times the expression in normal tissue.

The term "polynucleotide," when used in the singular or plural, generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. This includes, without limitation, single- and double-stranded DNA, DNA including single- and double- stranded regions, single-and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. Also included are triple-stranded regions comprising RNA or DNA or both RNA and DNA. Specifically included are mRNAs, cDNAs, and genomic DNAs, and any fragments thereof. The term includes DNAs and RNAs that contain one or more modified bases, such as tritiated bases, or unusual bases, such as inosine. The polynucleotides of the invention can encompass coding or non-coding sequences, or sense or antisense sequences. It will be understood that each reference to a "polynucleotide" or like term, herein, will include the full-length sequences as well as any fragments, derivatives, or variants thereof.

"Polypeptide," as used herein, refers to an oligopeptide, peptide, or protein sequence, or fragment thereof, and to naturally occurring, recombinant, synthetic, or semi-synthetic molecules. Where "polypeptide" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "polypeptide" and like terms, are not meant to limit the amino acid sequence to the complete, native amino acid sequence for the full-length molecule. It will be understood that each reference to a "polypeptide" or like term, herein, will include the full-length sequence, as well as any fragments, derivatives, or variants thereof.

The term "qPCR" or "QPCR" refers to quantitative polymerase chain reaction as described, for example, in PCR Technique: Quantitative PCR, J.W. Larrick, ed., Eaton Publishing, 1997, and A-Z of Quantitative PCR, S. Bustin, ed., IUL Press, 2004.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridisable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. Additional details and explanation of stringency of hybridization reactions, are found e.g., in Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, typically: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ a denaturing agent during hybridization, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate, buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X, Denhardt's solution, sonicated salmon sperm DNA (50 ug/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash comprising 0.1X SSC containing EDTA at 55°C.

"Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e. g., temperature, ionic strength, and % SDS) less stringent than those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1X SSC at about 37-50°C. The skilled artisan will

recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

The term "IL8Rb" means neutrophil marker interleukin 8 receptor B (also known as chemokine (C-X-C motif) receptor 2 [CXCR2]) (Figure 1; Seq ID Nos 1 and 2), and includes the marker IL8Rb. The term includes a polynucleotide, such as a gene or gene fragment, RNA or RNA fragment; or a gene product, including a polypeptide such as a peptide, oligopeptide, protein, or protein fragment; or any related metabolites, by products, or any other identifying molecules, such as antibodies or antibody fragments.

The term "reliability" includes the low incidence of false positives and/or false negatives. Thus, with higher reliability of a marker, fewer false positives and/or false negatives are associated with diagnoses made using that marker. Therefore, in certain embodiments, markers are provided that permit detection of bladder inflammatory disease or cancer with reliability greater than the reliability of prior art markers greater than 50%. In other embodiments, markers are provided that have reliability greater than about 70%; in other embodiments, greater than about 73%, in still other embodiments, greater than about 80%, in yet further embodiments, greater than about 90%, in still others, greater than about 95%, in yet further embodiments greater than about 98%, and in certain embodiments, about 100% reliability.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, and biochemistry, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, 2nd edition, Sambrook et al., 1989; *Oligonucleotide Synthesis*, MJ Gait, ed., 1984; *Animal Cell Culture*, R.I. Freshney, ed., 1987; *Methods in Enzymology*, Academic Press, Inc.; *Handbook of Experimental Immunology*, 4th edition, D .M. Weir & CC. Blackwell, eds., Blackwell Science Inc., 1987; *Gene Transfer Vectors for Mammalian Cells*, J.M. Miller & M.P. Calos, eds., 1987; *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds., 1987; and *PCR: The Polymerase Chain Reaction*, Mullis et al., eds., 1994.

It is to be understood that the above terms may refer to protein, DNA sequence and/or RNA sequence. It is also to be understood that the above terms also refer to non-human proteins, DNA and/or RNA having homologous sequences as depicted herein.

DESCRIPTION OF EMBODIMENTS OF THE INVENTION

Gene markers can be used as diagnostic tools to detect disease in a patient. The markers can, for example, be differentially expressed between disease tissue and corresponding non-disease tissue. In this situation, the detection of differential expression is associated with the presence of the disease. Alternatively, the marker can be associated directly with changes occurring in the disease tissues, or changes resulting from the disease. Inflammatory diseases are associated with an increase in neutrophils. It has been found that the neutrophil marker interleukin 8 receptor B (IL8Rb), (also known as chemokine (C-X-C motif) receptor 2 [CXCR2]) (Figure 1; Seq ID Nos 1 and 2), provides a good marker for the presence of neutrophils in a sample, and therefore can be used as a diagnostic marker for the detection of inflammatory disease in a sample, and in particular, in the detection of inflammatory disease of the bladder.

As shown in Figure 5, accumulation of IL8Rb in urine is indicative of the presence of inflammatory disease of the bladder. Specifically, Figure 5 shows the accumulation of IL8Rb in the urine of patients having the conditions; benign prostatic hyperplasia, urinary tract infection, non-specific prostate diseases, vascular prostate and secondary warfarin use. It will be appreciated however, that the use of IL8Rb is not be limited to the detection of these diseases only, but that these examples show that IL8Rb does increase in samples from patients having an inflammatory disease of the bladder. That is, IL8Rb can be used as a marker of inflammation associated with bladder disease and therefore is suitable for use in detecting any condition associated with inflammation. Therefore, the detection of the amount of IL8Rb can be used as a marker for inflammatory disease of the bladder. More particularly, IL8Rb can be used to detect inflammatory disease of the bladder associated with the accumulation of neutrophils.

Urine tests for transitional cell carcinoma of the bladder (TCC) rely largely on the presence of markers in exfoliated tumour cells in the urine. The ability to detect these cells can be masked by the presence of large numbers of contaminating cells, such as, blood and inflammatory cells. Moreover, inflammation of the bladder lining can result in the increased exfoliation of non-malignant cells from the mucosa. As a result, urine tests that use markers derived from bladder transitional cells have a higher likelihood of giving a false positive result from urine samples taken from patients with cystitis, urinary tract infection or other conditions resulting in urinary tract inflammation or transitional cell exfoliation, such as, urolithiasis (Sanchez-Carbayo et al).

One way to try and avoid such false positive results has been to select markers with low relative expression in blood or inflammatory cells. The use of such markers results in fewer false positives in TCC patients presenting with non-malignant, inflammatory conditions. However, low expression of the markers in haematologically-derived cells fails to compensate for the enhanced rate of exfoliation of non-malignant transitional cells.

It has been discovered that the negative impact of exfoliated transitional cells from inflamed tissue has on the accuracy of bladder cancer urine tests can be minimised by improving the identification of patients with inflammatory conditions of the urinary tract. Here it has been surprisingly found that using the marker IL8Rb in combination with one or more bladder tumour markers (BTM's) provides for a more accurate detection of bladder cancer. In particular, a marker based test for bladder cancer that includes the marker IL8Rb is less susceptible to false positive results, which can result in patients suffering from an inflammatory non-cancer condition.

In general the presence or absence of an inflammatory condition is established by having a threshold of gene expression, above which expression of IL8Rb is indicative of an inflammatory condition. For example, the expression of IL8Rb above a certain threshold is diagnostic that the patient has an inflammatory condition. When IL8Rb is used in conjunction with one or more markers predictive for the presence of bladder cancer, the presence of elevated expression of the bladder tumour marker(s), and expression of IL8Rb, above a certain threshold, is predictive of the patient having an inflammatory condition and not cancer. Furthermore, if the test is performed on urine from the patient, then this result is predictive of the patient having an inflammatory bladder condition. The high levels of the bladder tumour markers are most likely the result of non-malignant cells coming from the mucosa as a result of the inflammation. That is, the patient, although having high levels of the bladder tumour marker(s) does not actually have bladder cancer – a false positive.

Alternatively, if the patient has high levels or diagnostic levels of one or more bladder tumour markers, but the level of IL8Rb is below a threshold, then this is diagnostic that the patient is likely to have cancer, and in particular bladder cancer. This is especially so, if the test is performed on urine from the patient. This result is of significant benefit to the health provider because they can be sure that the patient does have cancer, and can start treatment immediately, and not be concerned that the result is actually caused an inflammatory condition giving a false positive result.

It has been surprisingly shown that the quantification of RNA from the gene encoding the neutrophil marker interleukin 8 receptor B (IL8Rb) improves the overall performance of detecting patients with TCC, using known TCC or BTM markers. The reference sequences for IL8Rb (also known as chemokine (C-X-C motif) receptor 2 [CXCR2]) are shown in Figure 1 and Seq ID Nos 1 and 2). In addition to its role in TCC detection, it has been explored whether IL8Rb could be used as a urine marker to aid in the diagnosis of inflammatory disease (Figure 5).

The novel use of IL8Rb marker can be used in isolation for the detection of inflammatory conditions of the bladder utilizing known methods for detecting gene expression levels. Examples of methods for detecting gene expression are outlined below.

Alternatively, IL8Rb can be combined with one of more BTMs to detect bladder cancer. It has been shown that by utilizing the novel inflammatory disease marker IL8Rb as part of the test for bladder cancer, the influence of inflamed tissue on creating a false positive result is minimized. The marker IL8Rb can be used in association with any bladder cancer markers, or alternatively can be used with two or more markers, as part of a signature, for detecting bladder cancer.

The action of IL8Rb to improve the detection of bladder cancer results from the ability to separate non-malignant conditions from patients having bladder cancer. This is achieved because an increase of IL8Rb is indicative of an increase in the presence of neutrophils in a sample. Therefore, the ability of IL8Rb is not dependant on the bladder tumour marker used. As shown in Figures 2, and 12 to 15, when combined with a variety of bladder tumour markers and combinations of bladder tumour markers, IL8Rb had the general effect of increasing the specificity of the ability of the marker(s) to detect cancer in the subjects.

It will be appreciated that any marker that is cable of detecting bladder cancer is suitable for use in combination with IL8Rb. Examples of known BTMs suitable for use in combination with IL8Rb in the detection of TCC are outlined Figures 6 and 7. Specifically, Figure 6 outlines markers known to be over expressed in bladder cancer, and Figure 7 outlines a number of markers known to be under expressed in bladder cancer. The novel use of the marker, IL8Rb of the present invention can be used in combination with any one or more of the markers of Figure 6 or Figure 7, or alternatively in combination with a signature comprising two or more markers selected from Figure 6 or Figure 7.

One example of a signature according to the present invention is the use of IL8Rb in combination with MDK, CDC2, IGFBP5 and HOXA13, which may also be in combination with one or more other marker suitable for detecting bladder cancer, for example any one of more of the markers outlined in Figure 6 or Figure 7. As shown in Figures 14 and 15 IL8Rb can be used in any combination of the markers, specifically the combinations IL8Rb / MDK, IL8Rb / CDC2, IL8Rb / HOXA13, IL8Rb / IGFBP5, IL8Rb / MDK / CDC2, IL8Rb / MDK / HOXA13, IL8Rb / MDK / IGFBP5, IL8Rb / CDC2 / HOXA13, IL8Rb / CDC2 / IGFBP5, IL8Rb / HOXA13 / IGFBP5, IL8Rb / MDK / CDC2 / HOXA13, IL8Rb / MDK / CDC2 / IGFBP5, IL8Rb / CDC2 / HOXA13 / IGFBP5, and IL8Rb / MDK / CDC2 / HOXA13 / IGFBP5. As shown in Figures 14 and 15, the inclusion of IL8Rb increased the ability of the marker, or the combination of markers to accurately diagnose bladder cancer in a subject. The present invention is not to be limited to these specific combinations but can optionally include one or more further markers suitable for detecting bladder cancer, for example any one of more of the markers outlined in Figure 6 or Figure 7.

PE Gene Name	HGNC Gene Name (Official)	NCBI RefSeq	NCBI Entrez Gene ID	HGNC URL
MDK	MDK	NM_002391	4192	http:// www.genenames.org/data/hgnc_data.php?hgnc_id=6972
CDC	CDK1	NM_001170406	983	http:// www.genenames.org/data/hgnc_data.php?hgnc_id=1722
IGF	IGFBP5	NM_000599	3488	http:// www.genenames.org/data/hgnc_data.php?hgnc_id=5474
HOXA	HOXA13	NM_000522	3209	http:// www.genenames.org/data/hgnc_data.php?hgnc_id=5102
IL8Rb	CXCR2	NM_001168298	3579	http:// www.genenames.org/data/hgnc_data.php?hgnc_id=6027

Table 1, shows the identifiers for the specific markers MDK, CDC2, IGFBP5 and HOXA13 and IL8Rb.

Figures 2 to 4, and 12 to 17 show the effect of using IL8Rb in combination with four known, representative, markers of bladder cancer; MDK, CDC2, IGFBP5 and HOXA13. The results show that by incorporating the use if IL8Rb individually with each marker (Figures 2, 14 and 15 to 17), but also when used with all possible combinations of the four BTM markers as a signature, there is an improvement in the ability to separate the samples of patients with TCC and those with non malignant conditions.

More specifically, as shown in Figures 10 to 13, the inclusion of IL8Rb with the four markers MDK, CDC2, IGFBP5 and HOXA13 (uRNA-D not only increased the overall

performance of the test compared to the four markers alone, the test also compared extremely favorably with other known tests, NMP22® “a registered trademark of Matritech, Inc., of Massachusetts, United States” Elisa, NMP22 BladderChek® “a registered trademark of Matritech, Inc., of Massachusetts, United States”, and cytology. Figures 14 through 17 also show the effect of IL8Rb in the various combinations of the four markers MDK, CDC2, IGFBP5 and HOXA13.

Specifically, Figure 14 shows the ROC curves for all the combinations of the four markers MDK, CDC2, IGFBP5 and HOXA13, with and without IL8Rb, calculated using five different classifier models (i) Linear Discriminant Analysis (LDA), (ii) Logistic Regression (LogReg), (iii) Support Vector Machines (SVM), (iv) K-nearest 5 neighbors (KN5N), and (v) Partition Tree Classifier (TREE). Figure 15 tabulates the Area Under the Curve (AUC) for all 5 classifiers and all 15 combinations of the 4 biomarkers, with and without IL8Rb. This AUC calculation is restricted to the area from a false positive rate of 0 to a false positive rate of 20%, covering the useful ranges of specificity (80 – 100%). The AUC quantifies the visible differences on the ROC curves of Figure 14. Figure 16 shows the sensitivity of all combinations of the four markers measured with and without IL8Rb at specificities of (a) 80%, (b) 85%, (c) 90%, (d) 95%, and (e) 98%. Figure 17 tabulates the changes in either sensitivity (vertical direction on the ROC curves; better is “up”) or specificity (horizontal direction on the ROC curve; better is to the left) at the fixed specificities of (a, f) 80%, (b, g) 85%, (c, h) 90%, (d, i) 95%, and (e, j) 98%.

These tables show that IL8Rb, in general, improves the ability of the biomarkers (MDK, CDC, IGFBP5, and HOXA13), singly or in combination, to classify tumour from normal samples.

These results generally show that the IL8Rb was able to increase the accuracy at which the test could detect bladder cancer. The biggest gains were seen with either markers that did not perform as well without the inclusion of IL8Rb or with classifiers that did not perform as well. Smaller gains were seen for markers and/or classifiers that performed well prior to adding IL8Rb and therefore there was less room for improvement. It is important to note that the results show a population based analysis and the benefit of incorporating IL8Rb could be greater when diagnosis individual patients, especially those whose diagnosis on the expression of the BTM markers maybe unclear.

These results show that not only can IL8Rb be used to detect inflammatory disease of

the bladder, but also when used in combination with markers for bladder cancer, results in an improved detection of bladder cancer, arising from a reduction in "false positive" results.

These results also show the utility of IL8Rb in that it affects the overall performance of the various markers combinations, and confirms the ability of IL8Rb to improve the performance of one or more bladder cancer markers to accurately detect cancer in a patient. Further, Figures 14 and 15 shows that the same results can be achieved using a range of classifier models, and shows that the result is not dependent on a classifier model or algorithm, but rather the combination of markers used. These results confirm that any suitable classifier model or algorithm could be used in the present invention. In particular, Figures 14 and 15 show that IL8Rb has a greater effect at the higher specificities, and in particular in the most clinically applicable ranges.

The present invention is based on the finding that detection of elevated levels of IL8Rb at either the polynucleotide or protein level in the urine is indicative of an inflammatory bladder disease. This can be used as a diagnosis tool alone for establishing an inflammatory bladder disease in a subject, but can also be used in conjunction with a test for bladder cancer in order to increase the specificity of the test by allowing the differentiation of the true positive result (ie the subject testing positive having bladder) from the false positive result (ie the subject testing positive for bladder cancer but in fact having an inflammatory bladder disease).

The method can be conducted on any suitable sample from the body that would be indicative of the urine, but ideally the level of IL8Rb, and any further cancer marker is established directly from a urine sample.

Detection of Markers in Body Samples

In several embodiments, assays for cancer can be desirably carried out on samples obtained from blood, plasma, serum, peritoneal fluid obtained for example using peritoneal washes, or other body fluids, such as urine, lymph, cerebrospinal fluid, gastric fluid or stool samples. For the detection of inflammatory conditions of the bladder or bladder cancer the test is ideally preformed on a urine sample.

Specifically the present method for detecting inflammatory bladder disease or bladder cancer can be conducted on any suitable sample from the body that would be indicative of

the urine, but ideally the level of IL8Rb, and any further cancer marker is established directly from a urine sample.

A test can either be preformed directly on a urine sample, or the sample may be stabilised by the addition of any suitable compounds or buffers known in the art to stabilise and prevent the breakdown of RNA and/or protein in the sample so that it can be analysed at a later date, or even to ensure that the RNA and/or protein is stabilized during the analysis.

The determination of either the protein and/or RNA level in the subject's urine can be preformed directly on the urine, or the urine can be treated to further purify and/or concentrate the RNA and/or protein. Many methods for extracting and/or concentrating proteins and/or RNA are well known in the art and could be used in the present invention.

In order to establish whether a particular subject has differential expression of IL8Rb and if necessary any further cancer marker, the level of either the RNA and/or protein of IL8Rb and optionally one or more cancer markers can be measured in the sample. It will be appreciated that many methods are well known in the art for establishing the level of a particular gene, either at the RNA and/or protein level, and any suitable method can be used in the present invention. Some common methods are outlined below, however, the invention is not restricted to these methods and any method for quantifying protein and/or RNA levels is suitable for use in the present invention.

General Approaches to Disease and Cancer Detection Using Gene Markers

General methodologies for determining expression levels are outlined below, although it will be appreciated that any method for determining expression levels would be suitable.

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) can be carried out on tumour samples, on serum and plasma using specific primers and probes. In controlled reactions, the amount of product formed in a PCR reaction (Sambrook, J., E Fritsch, E. and T Maniatis, Molecular Cloning: A Laboratory Manual 3rd. Cold Spring Harbor Laboratory Press: Cold Spring Harbor (2001)) correlates with the amount of starting template. Quantification of the PCR product can be carried out by stopping the PCR reaction when it is in log phase, before reagents become limiting. The PCR products are then electrophoresed in agarose or polyacrylamide gels, stained with ethidium bromide or a comparable DNA stain, and the intensity of staining measured by densitometry. Alternatively, the progression of a PCR reaction can be measured using PCR

machines such as the Applied Biosystems' Prism 7000™ “a trademark of Applied Biosystems Corporation, Connecticut, United States” or the Roche LightCycler™ (a trademark of Roche Molecular Systems, Inc., California, United States) which measure product accumulation in real-time. Real-time PCR measures either the fluorescence of DNA intercalating dyes such as Sybr Green into the synthesized PCR product, or the fluorescence released by a reporter molecule when cleaved from a quencher molecule; the reporter and quencher molecules are incorporated into an oligonucleotide probe which hybridizes to the target DNA molecule following DNA strand extension from the primer oligonucleotides. The oligonucleotide probe is displaced and degraded by the enzymatic action of the Taq polymerase in the next PCR cycle, releasing the reporter from the quencher molecule. In one variation, known as Scorpion, the probe is covalently linked to the primer.

Reverse Transcription PCR (RT-PCR)

RT-PCR can be used to compare RNA levels in different sample populations, in normal and tumour tissues, with or without drug treatment, to characterize patterns of expression, to discriminate between closely related RNAs, and to analyze RNA structure.

For RT-PCR, the first step is the isolation of RNA from a target sample. The starting material is typically total RNA isolated from human tumours or tumour cell lines, and corresponding normal tissues or cell lines, respectively. RNA can be isolated from a variety of samples, such as tumour samples from breast, lung, colon (e.g., large bowel or small bowel), colorectal, gastric, esophageal, anal, rectal, prostate, brain, liver, kidney, pancreas, spleen, thymus, testis, ovary, uterus, bladder etc., tissues, from primary tumours, or tumour cell lines, and from pooled samples from healthy donors. If the source of RNA is a tumour, RNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g., formalin-fixed) tissue samples.

The first step in gene expression profiling by RT-PCR is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. The two most commonly used reverse transcriptases are avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). The reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the circumstances and the goal of expression profiling. For example, extracted RNA can be reverse-transcribed using a GeneAmp® RNA PCR kit (Perkin Elmer, CA, USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction.

Although the PCR step can use a variety of thermostable DNA-dependent DNA polymerases, it typically employs the Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease activity. Thus, TaqMan® qPCR (a registered trademark of Roche Molecular Systems, Inc., California, United States) typically utilizes the 5' nuclease activity of Taq or Tth polymerase to hydrolyze a hybridization probe bound to its target amplicon, but any enzyme with equivalent 5' nuclease activity can be used.

Two oligonucleotide primers are used to generate an amplicon typical of a PCR reaction. A third oligonucleotide, or probe, is designed to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative interpretation of the data.

TaqMan® RT-PCR (a registered trademark of Roche Molecular Systems, Inc., California, United States) can be performed using commercially available equipment, such as, for example, ABI PRISM 7700™ Sequence Detection System (a trademark of Applied Biosystems, Foster City, CA, USA), or Lightcycler™ (a registered trademark of Roche Molecular Systems, Inc., California, United States) (Roche Molecular Biochemicals, Mannheim, Germany). In a preferred embodiment, the 5' nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700™ "a trademark of Applied Biosystems, Foster City, CA, USA" Sequence Detection System. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera, and computer. The system amplifies samples in a 96-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real-time through fibre optics cables for all 96 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

5' nuclease assay data are initially expressed as C_p , or the threshold cycle. As discussed above, fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The point when the fluorescent signal is first recorded as statistically significant is the threshold cycle.

Real-time Quantitative PCR (qRT-PCR)

A more recent variation of the RT-PCR technique is the real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan[®] probe (a registered trademark of Roche Molecular Systems, Inc., California, United States)). Real time PCR is compatible both with quantitative competitive PCR and with quantitative comparative PCR. The former uses an internal competitor for each target sequence for normalization, while the latter uses a normalization gene contained within the sample, or a housekeeping gene for RT-PCR. Further details are provided, e.g., by Held et al., *Genome Research* 6: 986-994 (1996).

Expression levels can be determined using fixed, paraffin-embedded tissues as the RNA source. According to one aspect of the present invention, PCR primers are designed to flank intron sequences present in the gene to be amplified. In this embodiment, the first step in the primer/probe design is the delineation of intron sequences within the genes. This can be done by publicly available software, such as the DNA BLAT software developed by Kent, W. J., *Genome Res.* 12 (4): 656-64 (2002), or by the BLAST software including its variations. Subsequent steps follow well established methods of PCR primer and probe design.

In order to avoid non-specific signals, it is useful to mask repetitive sequences within the introns when designing the primers and probes. This can be easily accomplished by using the Repeat Masker program available on-line through the Baylor College of Medicine, which screens DNA sequences against a library of repetitive elements and returns a query sequence in which the repetitive elements are masked. The masked sequences can then be used to design primer and probe sequences using any commercially or otherwise publicly available primer/probe design packages, such as Primer Express (Applied Biosystems); MGB assay-by-design (Applied Biosystems); Primer3 (Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the VIMNV for general users and for biologist programmers in: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386).

The most important factors considered in PCR primer design include primer length, melting temperature (T_m), and G/C content, specificity, complementary primer sequences, and 3' end sequence. In general, optimal PCR primers are generally 17-30 bases in length, and contain about 20-80%, such as, for example, about 50-60% G+C bases. Melting temperatures between 50 and 80°C, e.g., about 50 to 70°C, are typically preferred. For further guidelines for PCR primer and probe design see, e.g., Dieffenbach, C. W. et al., *General Concepts for PCR Primer Design in: PCR Primer, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1995, pp.133-155; Innis and Gelfand, *Optimization of PCRs in: PCR Protocols, A Guide to Methods and Applications*, CRC Press, London, 1994, pp. 5-11; and Plasterer, T. N. *Primerselect: Primer and probe design. Methods Mol. Biol.* 70: 520-527 (1997), the entire disclosures of which are hereby expressly incorporated by reference.

Microarray Analysis

Differential expression can also be identified, or confirmed using the microarray technique. Thus, the expression profile of disease specific markers can be measured in either fresh or paraffin-embedded tumour tissue, using microarray technology. In this method, polynucleotide sequences of interest (including cDNAs and oligonucleotides) are plated, or arrayed, on a microchip substrate. The arrayed sequences (i.e., capture probes) are then hybridized with specific polynucleotides from cells or tissues of interest (i.e., targets). Just as in the RT-PCR method, the source of RNA typically is total RNA isolated from human tumours or tumour cell lines, and corresponding normal tissues or cell lines. Thus RNA can be isolated from a variety of primary tumours or tumour cell lines. If the source of RNA is a primary tumour, RNA can be extracted, for example, from frozen or archived formalin fixed paraffin-embedded (FFPE) tissue samples and fixed (e.g., formalin-fixed) tissue samples, which are routinely prepared and preserved in everyday clinical practice.

In a specific embodiment of the microarray technique, PCR amplified inserts of cDNA clones are applied to a substrate. The substrate can include up to 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 75 nucleotide sequences. In other aspects, the substrate can include at least 10,000 nucleotide sequences. The microarrayed sequences, immobilized on the microchip, are suitable for hybridization under stringent conditions. As other embodiments, the targets for the microarrays can be at least 50, 100, 200, 400, 500, 1000, or 2000 bases in length; or 50-100, 100-200, 100-500, 100-1000, 100-2000, or 500-5000 bases in length. As further embodiments, the capture probes for the microarrays can be at least 10, 15, 20, 25,

50, 75, 80, or 100 bases in length; or 10-15, 10-20, 10-25, 10-50, 10-75, 10-80, or 20-80 bases in length.

Fluorescently labeled cDNA probes may be generated through incorporation of fluorescent nucleotides by reverse transcription of RNA extracted from tissues of interest. Labeled cDNA probes applied to the chip hybridize with specificity to each spot of DNA on the array. After stringent washing to remove non-specifically bound probes, the chip is scanned by confocal laser microscopy or by another detection method, such as a CCD camera. Quantitation of hybridization of each arrayed element allows for assessment of corresponding mRNA abundance. With dual colour fluorescence, separately labeled cDNA probes generated from two sources of RNA are hybridized pairwise to the array. The relative abundance of the transcripts from the two sources corresponding to each specified gene is thus determined simultaneously.

The miniaturized scale of the hybridization affords a convenient and rapid evaluation of the expression pattern for large numbers of genes. Such methods have been shown to have the sensitivity required to detect rare transcripts, which are expressed at a few copies per cell, and to reproducibly detect at least approximately two-fold differences in the expression levels (Schena et al., *Proc. Natl. Acad. Sci. USA* 93 (2): 106-149 (1996)). Microarray analysis can be performed by commercially available equipment, following manufacturer's protocols, such as by using the Affymetrix GenChip technology, Illumina microarray technology or Incyte's microarray technology. The development of microarray methods for large-scale analysis of gene expression makes it possible to search systematically for molecular markers of cancer classification and outcome prediction in a variety of tumour types.

RNA Isolation, Purification, and Amplification

General methods for mRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al., *Current Protocols of Molecular Biology*, John Wiley and Sons (1997). Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker, *Lab Invest.* 56: A67 (1987), and De Sandres et al., *BioTechniques* 18: 42044 (1995). In particular, RNA isolation can be performed using purification kit, buffer set, and protease from commercial manufacturers, such as Qiagen, according to the manufacturer's instructions. For example, total RNA from cells in culture can be isolated using Qiagen RNeasy® "a registered trademark of Qiagen GmbH, Hilden, Germany" mini-columns. Other commercially available RNA isolation kits include MasterPure Complete DNA and RNA Purification Kit (EPICENTRE

(D, Madison, WI), and Paraffin Block RNA Isolation Kit (Ambion, Inc.). Total RNA from tissue samples can be isolated using RNA Stat-60 (Tel-Test). RNA prepared from tumour can be isolated, for example, by cesium chloride density gradient centrifugation.

The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: T. E. Godfrey et al. *J. Molec. Diagnostics* 2: 84-91 (2000); K. Specht et al., *Am. J. Pathol.* 158: 419-29 (2001)). Briefly, a representative process starts with cutting about 10 micron thick sections of paraffin-embedded tumour tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by RT-PCR. Finally, the data are analyzed to identify the best treatment option(s) available to the patient on the basis of the characteristic gene expression pattern identified in the tumour sample examined.

Immunohistochemistry and Proteomics

Immunohistochemistry methods are also suitable for detecting the expression levels of the proliferation markers of the present invention. Thus, antibodies or antisera, preferably polyclonal antisera, and most preferably monoclonal antibodies specific for each marker, are used to detect expression. The antibodies can be detected by direct labeling of the antibodies themselves, for example, with radioactive labels, fluorescent labels, hapten labels such as, biotin, or an enzyme such as horseradish peroxidase or alkaline phosphatase. Alternatively, unlabeled primary antibody is used in conjunction with a labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal antibody specific for the primary antibody. Immunohistochemistry protocols and kits are well known in the art and are commercially available.

Proteomics can be used to analyze the polypeptides present in a sample (e.g., tissue, organism, or cell culture) at a certain point of time. In particular, proteomic techniques can be used to assess the global changes of polypeptide expression in a sample (also referred to as expression proteomics). Proteomic analysis typically includes: (1) separation of individual polypeptides in a sample by 2-D gel electrophoresis (2-D PAGE); (2) identification of the individual polypeptides recovered from the gel, e.g., by mass spectrometry or N-terminal sequencing, and (3) analysis of the data using bioinformatics. Proteomics methods are

valuable supplements to other methods of gene expression profiling, and can be used, alone or in combination with other methods, to detect the products of the proliferation markers of the present invention.

Hybridization Methods Using Nucleic Acid Probes Selective for a Marker

These methods involve binding the nucleic acid probe to a support, and hybridizing under appropriate conditions with RNA or cDNA derived from the test sample (Sambrook, J., E Fritsch, E. and T Maniatis, *Molecular Cloning: A Laboratory Manual* 3rd. Cold Spring Harbor Laboratory Press: Cold Spring Harbor (2001)). These methods can be applied to markers derived from a tumour tissue or fluid sample. The RNA or cDNA preparations are typically labeled with a fluorescent or radioactive molecule to enable detection and quantification. In some applications, the hybridizing DNA can be tagged with a branched, fluorescently labeled structure to enhance signal intensity (Nolte, F.S., Branched DNA signal amplification for direct quantitation of nucleic acid sequences in clinical specimens. *Adv. Clin. Chem.* 33, 201-35 (1998)). Unhybridized label is removed by extensive washing in low salt solutions such as 0.1x SSC, 0.5% SDS before quantifying the amount of hybridization by fluorescence detection or densitometry of gel images. The supports can be solid, such as nylon or nitrocellulose membranes, or consist of microspheres or beads that are hybridized when in liquid suspension. To allow washing and purification, the beads may be magnetic (Haukanes, B-1 and Kvam, C., Application of magnetic beads in bioassays. *Bio/Technology* 11, 60-63 (1993)) or fluorescently-labeled to enable flow cytometry (see for example: Spiro, A., Lowe, M. and Brown, D., A Bead-Based Method for Multiplexed Identification and Quantitation of DNA Sequences Using Flow Cytometry. *Appl. Env. Micro.* 66, 4258-4265 (2000)).

A variation of hybridization technology is the QuantiGene Plex[®] assay (a registered trademark of Panomics, of California, United States) (Genospectra, Fremont) which combines a fluorescent bead support with branched DNA signal amplification. Still another variation on hybridization technology is the Quantikine[®] mRNA assay (R&D Systems, Minneapolis). Methodology is as described in the manufacturer's instructions. Briefly the assay uses oligonucleotide hybridization probes conjugated to Digoxigenin. Hybridization is detected using anti-Digoxigenin antibodies coupled to alkaline phosphatase in colorimetric assays.

Additional methods are well known in the art and need not be described further herein.

Enzyme-Linked Immunological Assays (ELISA)

Briefly, in sandwich ELISA assays, a polyclonal or monoclonal antibody against the marker is bound to a solid support (Crowther, J.R. *The ELISA guidebook*. Humana Press: New Jersey (2000); Harlow, E. and Lane, D., *Using antibodies: a laboratory manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor (1999)) or suspension beads. Other methods are known in the art and need not be described herein further. Monoclonal antibodies can be hybridoma-derived or selected from phage antibody libraries (Hust M. and Dubel S., *Phage display vectors for the in vitro generation of human antibody fragments*. *Methods Mol Biol*. 295:71-96 (2005)). Nonspecific binding sites are blocked with non-target protein preparations and detergents. The capture antibody is then incubated with a preparation of sample or tissue from the patient containing the antigen. The mixture is washed before the antibody/antigen complex is incubated with a second antibody that detects the target marker. The second antibody is typically conjugated to a fluorescent molecule or other reporter molecule that can either be detected in an enzymatic reaction or with a third antibody conjugated to a reporter (Crowther, Id.). Alternatively, in direct ELISAs, the preparation containing the marker can be bound to the support or bead and the target antigen detected directly with an antibody-reporter conjugate (Crowther, Id.).

Methods for producing monoclonal antibodies and polyclonal antisera are well known in the art and need not be described herein further.

Immunodetection

The methods can also be used for immunodetection of marker family members in sera or plasma from bladder cancer patients taken before and after surgery to remove the tumour, immunodetection of marker family members in patients with other cancers, including but not limited to, colorectal, pancreatic, ovarian, melanoma, liver, oesophageal, stomach, endometrial, and brain and immunodetection of marker family members in urine and stool from bladder cancer patients.

Disease markers can also be detected in tissues or samples using other standard immunodetection techniques such as immunoblotting or immunoprecipitation (Harlow, E. and Lane, D., *Using antibodies: a laboratory manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor (1999)). In immunoblotting, protein preparations from tissue or fluid containing the marker are electrophoresed through polyacrylamide gels under denaturing or non-denaturing conditions. The proteins are then transferred to a membrane support such as nylon. The marker is then reacted directly or indirectly with monoclonal or polyclonal

antibodies as described for immunohistochemistry. Alternatively, in some preparations, the proteins can be spotted directly onto membranes without prior electrophoretic separation. Signal can be quantified by densitometry.

In immunoprecipitation, a soluble preparation containing the marker is incubated with a monoclonal or polyclonal antibody against the marker. The reaction is then incubated with inert beads made of agarose or polyacrylamide with covalently attached protein A or protein G. The protein A or G beads specifically interact with the antibodies forming an immobilized complex of antibody-marker-antigen bound to the bead. Following washing the bound marker can be detected and quantified by immunoblotting or ELISA.

Establishing a Diagnosis

Once the level of expression of IL8Rb, and optionally one or more further cancer markers, has been obtained then a diagnosis for that subject can be established. If the expression of IL8Rb is above the expression seen in subjects that do not have an inflammatory bladder disease, and/or is consistent with the level of expression in subjects known to have an inflammatory bladder disease, then the subject will be diagnosed as having an inflammatory bladder disease. Alternatively, if the expression is not above the expression seen in subjects that do not have an inflammatory bladder disease, and/or is below the levels of expression in subjects known to have an inflammatory bladder disease, then the subject will be diagnosed as not an inflammatory bladder disease.

In the situation where IL8Rb is used in conjunction with one or more markers for Bladder cancer, then the expression level of IL8Rb will be compared with the level of expression of subjects without an inflammatory bladder disease, and/or subjects known to have an inflammatory bladder disease. The one or more cancer markers are compared to the expression level in subjects without bladder cancer and/or subjects known to have bladder cancer. If the expression level of the IL8Rb is consistent with a subject that does not have an inflammatory bladder disease (less than a subject having an inflammatory bladder disease) and the expression level of the one or more bladder cancer markers are consistent with a subject having bladder cancer (differential to a subject that does not have bladder cancer), then the subject is diagnosed as having bladder cancer. If the expression level of the IL8Rb is greater than a subject that does not have an inflammatory bladder disease (consistent with a subject having an inflammatory bladder disease) and the expression level of the one or more bladder cancer markers are consistent with a subject having bladder cancer (differential to a subject that does not have bladder cancer), then the subject is

diagnosed as having an inflammatory bladder disease. If the expression level of the IL8Rb is consistent with a subject that does not have an inflammatory bladder disease (less than a subject having an inflammatory bladder disease) and the expression level of the one or more bladder cancer markers are consistent with a subject that does not have bladder cancer (differential to a subject that does have bladder cancer), then the subject is diagnosed as having neither bladder cancer or an inflammatory bladder disease.

Because there is often an overlap in expression levels between the normal and disease expression of a diagnostic marker, in order to establish a diagnosis for a subject it is typical to establish a classifying threshold. A classifying threshold is a value or threshold which distinguishes subjects into disease or non disease categories. A threshold is commonly evaluated with the use of a Receiver Operating Characteristic (ROC) curve, which plots the sensitivity against specificity for all possible thresholds.

Diagnostic Threshold Determination

For tests using disease markers, diagnostic thresholds are derived that enable a sample to be called either positive or negative for the disease, e.g., bladder cancer. These diagnostic thresholds are determined by the analysis of cohorts of patients that are investigated for the presence of bladder cancer or inflammatory bladder disease. Diagnostic thresholds may vary for different test applications; for example, diagnostic thresholds for use of the test in population screening are determined using cohorts of patients who are largely free of urological symptoms, and these diagnostic thresholds may be different to those used in tests for patients who are under surveillance for bladder cancer recurrence. A diagnostic threshold is selected to provide a practical level of test specificity in the required clinical setting; that is, a specificity that allows reasonable sensitivity without excessive numbers of patients receiving false positive results. This specificity may be within the range of 80-100%..

A diagnostic threshold is determined by applying the algorithms that combines the gene expression levels of each marker to each sample from a prospective clinical trial. Samples used are from patients with bladder cancer and a range of non-malignant urological disorders. A diagnostic threshold is selected by determining the score of the algorithm that resulted in the desired specificity. For example, in some applications a specificity of 85% is desired. The A diagnostic threshold is then set by selecting an algorithm score that results in 85% of patients without bladder cancer being correctly classed as negative for cancer. In other applications (such as population screening), higher specificity, such as 90%, is

favoured. To set a threshold for this application, an algorithm score that results in 90% of patients without bladder cancer being correctly classed as negative for cancer is selected. Examples of the use of an algorithm is outlined in the Examples.

As an alternative to single thresholds, the test may use test intervals which provide different degrees of likelihood of presence of disease and which have different clinical consequences associated with them. For example, a test may have three intervals; one associated with a high (e.g. 90%) risk of the presence of bladder cancer, a second associated with a low risk of bladder cancer and a third regarded as being suspicious of disease. The "suspicious" interval could be associated with a recommendation for a repeat test in a defined period of time.

Data Analysis

Once the method to test for the amount of RNA and/or protein has been completed, the data then has to be analysed in order to determine the distribution of biomarker values associated with tumour and normal samples. . This typically involves normalizing the raw data, ie removing background "noise" etc and averaging any duplicates (or more), comparison with standards and establishing cut-offs or thresholds to optimally separate the two classes of samples. Many methods are known to do this, and the exact method will depend on specific method for determining the amount of RNA and/or protein used.

Below is an example of how the data analysis could be preformed when using qRT-PCR. However, it will be appreciated the general process could be adapted to be used for other methods of establishing the RNA and/or protein content, or other methods could be established by someone skilled in the art to achieve the same result.

Data

Measurements of fluorescence are taken at wavelengths ω_i $i=1,2$ at each cycle of the PCR.. Thus for each well we observe a pair of fluorescence curves, denoted by $f_t(\omega_i)$, where $t=1,\dots,k$ denotes cycle number and $i=1,2$ indexes the wavelengths.

Fluorescence curves have a sigmoidal shape beginning with a near horizontal baseline and increasing smoothly to an upper asymptote. The location of a point C_p where the

fluorescence curve departs from the linear baseline will be used to characterize the concentration of the target gene. A precise definition of C_p follows later.

The following is an example of a scheme to process these data.

- Compensate for fluorescence overlap between frequency bands,
- Estimate a smooth model for each fluorescence curve in order to estimate C_p
- Combine data from replicated wells.
- Estimate standard curves
- Compute a concentration relative to the standard.

Each biological sample yields relative concentrations of 5 genes, which are the inputs to the discriminant function.

Colour compensation

Denote the level of fluorescence of dye j at cycle t and frequency ω by $W_{jt}(\omega)$. In a multiplexed assay the measured response at any frequency ω is the sum of contributions from all dyes at that frequency, so for each cycle

$$f_t(\omega) = W_{t1}(\omega) + W_{t2}(\omega) + \dots$$

The purpose of colour compensation is to extract the individual contributions $W_{jt}(\omega)$, from the observed mixtures $f_t(\omega)$.

In the ideal situation, fluorescence $W_{jt}(\omega_o)$, due to dye j at a frequency ω is proportional to its fluorescence $W_{jt}(\omega_o)$ at reference frequency ω_o , regardless of the level of $W_{jt}(\omega_o)$. This suggests the linear relationship

$$\begin{bmatrix} f_t(\omega_1) \\ f_t(\omega_2) \end{bmatrix} = \begin{bmatrix} W_{t1}(\omega_1) + W_{t2}(\omega_1) \\ W_{t1}(\omega_2) + W_{t2}(\omega_2) \end{bmatrix} = \begin{bmatrix} 1 & A_{12} \\ A_{21} & 1 \end{bmatrix} \begin{bmatrix} W_{t1}(\omega_1) \\ W_{t2}(\omega_2) \end{bmatrix}$$

for some proportionality constants A_{12} and A_{21} that are to be determined. In reality, there are additional effects, which are effectively modelled by introducing linear terms in this system, so

$$\begin{bmatrix} f_t(\omega_1) \\ f_t(\omega_2) \end{bmatrix} = \begin{bmatrix} 1 & A_{12} \\ A_{21} & 1 \end{bmatrix} \begin{bmatrix} W_{t1}(\omega_1) \\ W_{t2}(\omega_2) \end{bmatrix} + \begin{bmatrix} a_1 + b_1 t \\ a_2 + b_2 t \end{bmatrix}$$

After estimating the “colour compensation” parameters A_{12} and A_{21} we can recover $W_{t1}(\omega_1)$ and $W_{t2}(\omega_2)$, albeit distorted by a linear baseline, by matrix multiplication:

$$\begin{bmatrix} W_{t1}(\omega_1) \\ W_{t2}(\omega_2) \end{bmatrix} = \begin{bmatrix} 1 & A_{12} \\ A_{21} & 1 \end{bmatrix}^{-1} \begin{bmatrix} f_t(\omega_1) \\ f_t(\omega_2) \end{bmatrix} + \begin{bmatrix} a_1^* + b_1^* t \\ a_2^* + b_2^* t \end{bmatrix}$$

The $W_{t1}(\omega_1)$ and $W_{t2}(\omega_2)$ will be called “colour compensated” data. The linear distortions $a_i^* + b_i^* t$ in the last term of this expression will be accommodated in the baseline estimate when estimating a model for the colour compensated data below 2. It has no influence on the estimate of C_p .

Estimation of the colour compensation coefficients requires a separate assay using single (as opposed to duplex) probes. Then $W_{t2}(\omega_2) = 0$ giving

$$\begin{bmatrix} f_t(\omega_1) \\ f_t(\omega_2) \end{bmatrix} = \begin{bmatrix} 1 & A_{12} \\ A_{21} & 1 \end{bmatrix} \begin{bmatrix} W_{t1}(\omega_1) \\ 0 \end{bmatrix} + \begin{bmatrix} a_1 + b_1 t \\ a_2 + b_2 t \end{bmatrix}$$

Thus

$$f_t(\omega_2) = A_{21} f_t(\omega_1) + a^* + b^* t$$

Now the coefficient A_{21} can be estimated by ordinary linear regression of $f_t(\omega_2)$ on $f_t(\omega_1)$ and PCR cycle t for $t = 1, \dots, k$.

Model Estimation

In this section, let $y_t, t=1, \dots, k$ denote a colour compensated fluorescence curve.

Amplification

Models are only estimated for fluorescence curves that show non-trivial amplification. We define the term “amplification” as a non-trivial departure from the linear baseline of the colour compensated fluorescence curve. Use signal to noise ratio (SNR) to quantify amplification. Here SNR is defined as the ratio of signal variance to noise variance. Noise variance is set as part of calibration of the assay procedure and remains unchanged: for this purpose, use the residual variance from a linear model for the baseline from wells that can have no amplification, i.e. wells without RNA. For each fluorescence curve, estimate the signal

variance as the residual variance from the best fitting straight line (Here “best” is meant in the least squares sense.)

- If SNR is less than a specified threshold, the fluorescence curve is close to linear and no amplification is present. Then there is no point of departure from the baseline and the concentration in the sample may be declared as zero.
- If the SNR is above the threshold, amplification is present and a concentration can be estimated.

Thresholds for the (dimensionless) SNR are selected to provide clear discrimination between “amplified” and “non amplified” curves. For example, the following ranges for thresholds are effective for the markers.

Fluor	Gene	Range
JOE	MDK	40-120 15
JOE	CDC	35-70
JOE	IL8R	30-60
FAM	IGF	50-80
FAM	HOXA	50-150
FAM	XENO	50-80 20

Model

Estimate a sigmoidal model for each fluorescence curve. Any suitable parametric form of model can be used, but it must be able to model the following features:

- linear baseline that may have a non-zero slope,
- asymmetries about the mid point.
- asymptotes at lower and upper levels
- smooth increase from baseline to upper asymptote

An example of a model that achieves these requirements is

$$g_t(\theta) = A + A_s t + \frac{D}{\left(1 + \left(\frac{t}{B}\right)^E\right)^F}$$

We call this the “6PL model”. The parameter vector $\theta = [A, A_s, D, B, E, F]$ is subject to the following constraints to ensure that $g_t(\theta)$ is an increasing function of t and has the empirical properties of a fluorescence curve.

$$D > 0, B > 0, E < 0, F < 0$$

The other two parameters determine the base line $A + A_s t$, and these parameters do not need explicit constraints though A is always positive and the slope parameter A_s is always small.

The parameter D determines the level of amplification above the baseline. The remaining parameters B, E, F have no intrinsic interpretation in themselves but control the shape of the curve. These parameters are also the only parameters that influence the estimate of C_p . When $A_s = 0$ this is known as the five-parameter logistic function (5PL) and if, in addition, $F = 1$ this model reduces four-parameter logistic model (4PL), Gottschalk and Dunn (2005), Spiess et al. (2008).

Initialization

Initial values for non-linear estimation are set as

- $A_s = 0, F = 1$
- $A = \text{mean}(y_1, \dots, y_5)$
- $D = \text{range}(y_1, \dots, y_k)$
- $B = \text{cycle corresponding to half height}$
- E is initialized by converting $g_t(\theta)$ into a linear form having set the values of the remaining parameters to their initial values defined above. Linearization obtains

$$E \log\left(1 + \frac{t}{B}\right) = \log\left(\frac{D}{y_t - A}\right)$$

Now estimate E by regression of $\log\left(\frac{D}{y_t - A}\right)$ on $\log\left(1 + \frac{t}{B}\right)$ for t selected so that

$$A + \frac{D}{10} < y_t < A + \frac{9D}{10}$$

An alternative form of this model that leads to an almost identical analysis (with its own initialization) is

$$A + A_s t + D / (1 + \exp(-(t - B)/E))^F$$

When $A_s = 0$ this is sometimes known as the Richards equation, Richards (1959)

Estimation criterion

Estimate parameters to minimize a penalized sum of squares criterion

$$\sum_t (y_t - g_t(\theta))^2 + \lambda(\theta)$$

Here $\lambda(\theta)$ is a non-negative function that penalizes large values of some (or all) of the parameters in θ . This method is known as regularization or ridge regression (Hoerl, 1962) and may be derived from a Bayesian viewpoint by setting a suitable prior distribution for the parameter vector θ . A satisfactory choice for the penalty is

$$\lambda(\theta) = \lambda(B^2 + D^2 + E^2 + F^2)$$

Large values of λ bias the parameter estimates towards zero and reduce the variance of the parameter estimates. Conversely, small (or zero) λ leads to unstable parameter estimates and convergence difficulties in minimization algorithms. The choice of λ is a compromise between bias and variance or stability. Empirical evidence shows that a satisfactory compromise between bias and variance may be achieved if λ is chosen in the range

$$0.01 > \lambda > 0.0001.$$

This choice also ensures convergence of the optimization algorithm.

Algorithm choice

For any choice of λ in the above range, the description in the previous paragraph completely defines the parameter estimates. A non-linear least squares procedure based on the classical Gauss-Newton procedure (such as the Levenberg-Marquardt algorithm as implemented in More, 1978) has been successfully used and is a suitable approach. General purpose optimizing algorithms such as Nelder and Mead, 1965, or Broyden-Fletcher

algorithm as implemented by Byrd, et al., 1995) have also been successfully trialed in this context.

C_p estimate

C_p is the point t that maximizes the second derivative of $g_t(\theta)$. Each fluorescence curve yields a C_p that characterizes the concentration of the target gene.

The average of the estimated C_p 's for each set of technical replicates is computed and used in the subsequent analysis.

1. Standard curves

Absolute or relative concentrations are derived from a comparison with standard curves on the same PCR plate.

Model dilution series using the linear model

$$C_p = R + S \log_{10} Conc$$

where $Conc$ is an absolute or relative concentration of the standard. The intercept and slope parameters are plate specific. Model the between-plate variability in the intercept and slope parameters by setting population models

$$R \sim N(\mu_R, \sigma_R^2)$$

$$S \sim N(\mu_S, \sigma_S^2)$$

where the parameters $\mu_R, \sigma_R^2, \mu_S, \sigma_S^2$ are set on the basis of prior data as described below. Then for a given plate R and S can be interpreted as observations from these populations.

For replicate i of standard at concentration $Conc_j$ the following model can be used

$$C_p(i, j) = R + S \log_{10} Conc_j + \epsilon_{ij}$$

where $\epsilon_{ij} \sim N(0, \sigma_j^2)$. Note that the variance of the residuals depends on C_p . Empirical estimates of $Var(\epsilon_{ij})$ are given in Table 2. Estimate the parameters R and S using by

maximizing the likelihood function. Interpret the slope parameter in terms of the efficiency of the PCR process through

$$S = -\frac{1}{\log_{10} \text{Efficiency}}$$

This model has a Bayesian interpretation: Give vague (non-informative) prior distributions to the parameters $\mu_R, \sigma_R^2, \mu_S, \sigma_S^2$. Then the population models for R and S and for $C_p(i,j)$ fully determine a probability model for the prior data. A Markov chain Monte Carlo (MCMC) algorithm (Lunn et al., 2009) allows estimation of $\mu_R, \sigma_R^2, \mu_S, \sigma_S^2$. If the prior distribution is omitted, a traditional frequentist interpretation results. Following this estimation procedure it is possible to obtain the gene-dependent population parameter estimates in Table 3.

Table 2: Variance of residuals

C_p	σ^2	C_p	σ^2
12	0.0100	21	0.0466
13	0.0108	22	0.0625
14	0.0119	23	0.0860
15	0.0134	24	0.1212
16	0.0155	25	0.1750
17	0.0184	26	0.2591
18	0.0224	27	0.3931
19	0.0279	28	0.6112
20	0.0356	29	0.9741

Table 3 Population parameters for slopes and intercepts of standard curves.

	μ_R	σ_R^2	μ_S	σ_S^2
MDK	19.49	0.5112	-3.426	0.0481
CDC	18.91	0.2343	-3.414	0.0198
IL8R	31.43	0.0919	-3.192	0.0017
IGF	20.63	0.3835	-3.275	0.0247
HOXA	22.51	0.1544	-3.270	0.0037

The estimates of intercept and slope of the standard curve are denoted by $\hat{R}$ and $\hat{S}$.

2. Relative concentrations ΔC_p

Use the standard curve to compute $C_{p(REF)}$ at the concentration $CONC_{REF}$ from

$$C_{p(REF)} = \hat{R} + \hat{S} \log_{10} CONC_{REF}$$

The relative concentration of a sample is given by

$$\Delta C_p = \frac{C_p - C_{p(REF)}}{\hat{S}} = \log_{10} \frac{CONC_{SAMPLE}}{CONC_{REF}}$$

Alternatively $\hat{S}$ may be approximated at a fixed level corresponding to a PCR efficiency of 2. Then $\hat{S} = -1/\log_{10}(2) = -3.32$. Use the same notation ΔC_p for either choice.

The resulting ΔC_p estimates, one for each gene, are inputs to the discriminant function in the next step.

3. Discriminant function

The ΔC_p values correspond to a relative biomarker value with plate-to-plate variation removed. Examination of the 5 ΔC_p values in comparison with each other (for example, see Figure 2), shows how tumor samples typically have different biomarker values than non-tumor samples. Furthermore, while there is overlap in the areas for tumor and normal, a large number of samples are effectively well separated. Under these circumstances, many different statistical classifiers could be used to separate the normal from the tumor samples. We show here that a sample of several classifiers do work to separate these samples. We used 5 different classification methods: 1) Linear Discriminant Analysis (LDA), 2) Logistic

Regression (LogReg); 3) Support Vector Machines (SVM); 4) K-nearest-neighbor (KNN) based on 5 neighbors (KN5N); and 5) Recursive partitioning trees (TREE) (Cite: Venables & Ripley and Dalgaard).

Creation of a classifier requires a dataset containing the biomarker values for a large number of samples which should represent the ultimate population to be tested by the classifier. For example, if a classifier is to be used for screening an at-risk population (eg age 50 and older, smokers), then the set of data required for creating the classifier (called the "training set") should mirror that population and contain only samples from people older than 50 who smoke. Typically to obtain measurement precision of smaller than 10% error for parameters like sensitivity and specificity, the training set needs to be larger than 300 samples.

Estimation of the effectiveness of a classifier can be made using cross-validation. In cross-validation ([Wikipedia: Cross-validation](#)), the dataset is divided into a small number of equally sized partitions (typically 3 to 10). One section is left out and the remaining sections used to build a classifier; then the left out section is tested by the new classifier and its predictions noted. This is done for each section in turn and all the predictions combined and analysed to compute the characteristics of the classifier: Sensitivity, Specificity, etc. If the cross-validation is performed by partitioning the data into 10 parts, it is called 10-fold cross-validation; similarly, 3 parts would be 3-fold cross-validation. If the data are partitioned into as many classes as there are samples, this is called "leave one out cross-validation". By testing on data not used to build the classifier, this method provides an estimate of the classifier performance in the absence of additional samples.

We have built classifiers using all 15 combinations of the 4 biomarkers, MDK, IGFBP5, CDC2, and HOXA13, all with and without the IL8Rb biomarker, using the clinical trial dataset described elsewhere in this document (Example 1) and tested those 30 classifiers using 10-fold cross-validation. This was done for each of the 5 classifier types listed above and the ROC curves computed. All work was performed using the R Statistical Programming Environment (CITE). These results (Figure 14) show that in most cases, the classifier with IL8Rb is more sensitive for values of specificity which are useful diagnostically (False Positive Rate of 0 to 20%; Specificity from 100 to 80%). The Area Under the Curve (AUC) for the region with diagnostic utility of specificities is used to quantify how well classifiers perform with larger values indicating better classifier performance. Figure 15a tabulates the AUC for each classifier and biomarker combination, while Figure 15b shows the amount of increase in

AUC for each condition when IL8Rb is added. In most cases, the addition of IL8Rb improves the ability to make accurate diagnoses. Specific sensitivity values for diagnostically useful specificity values are tabulated for all the classifiers in Figure 16. In addition, Figure 17 tabulates the amount of gain in sensitivity or specificity which the addition of IL8Rb provides.

The utility of the classifier is created when, having created it and tested it, it is used to test a new sample. To simplify the interpretation of results, a cut-off score or threshold is established; samples on one side of the cut-off are considered positive and on the other side, negative for tumors. Additional cut-offs may be established for example to indicate increasing levels of certainty of results. In this case, we have established a cut-off which gives a false positive rate of 15% in our training set. Using our cross-validated ROC curves, we can then estimate our sensitivity. Typically, we also establish a cut-off at a positive predictive value of 75%. To use these cut-offs we establish a "negative" result for scores less than the cut-off established by the 85% specificity. Scores greater than the 75% PPV are called "positive" and score between the two are called "indeterminate" or "suspicious".

Antibodies to IL8Rb Marker

In additional aspects, this invention includes manufacture of antibodies against IL8Rb. The marker IL8Rb can be produced in sufficient amount to be suitable for eliciting an immunological response. In some cases, a full-length IL8Rb can be used, and in others, a peptide fragment of a IL8Rb may be sufficient as an immunogen. The immunogen can be injected into a suitable host (e.g., mouse, rabbit, etc) and if desired, an adjuvant, such as Freund's complete adjuvant or Freund's incomplete adjuvant can be injected to increase the immune response. It can be appreciated that making antibodies is routine in the immunological arts and need not be described herein further. As a result, one can produce antibodies, including monoclonal or phage-display antibodies, against IL8Rb.

In yet further embodiments, antibodies can be made against the protein or the protein core of the tumour markers identified herein or against an oligonucleotide sequence unique to a IL8Rb. Although certain proteins can be glycosylated, variations in the pattern of glycosylation can, in certain circumstances, lead to mis-detection of forms of IL8Rb that lack usual glycosylation patterns. Thus, in certain aspects of this invention, IL8Rb immunogens can include deglycosylated IL8Rb or deglycosylated IL8Rb fragments. Deglycosylation can be accomplished using one or more glycosidases known in the art. Alternatively, IL8Rb cDNA can be expressed in glycosylation-deficient cell lines, such as prokaryotic cell lines, including E. coli and the like.

Vectors can be made having IL8Rb -encoding oligonucleotides therein. Many such vectors can be based on standard vectors known in the art. Vectors can be used to transfect a variety of cell lines to produce IL8Rb -producing cell lines, which can be used to produce desired quantities of IL8Rb for development of specific antibodies or other reagents for detection of IL8Rb or for standardizing developed assays for IL8Rb.

Kits

Based on the discoveries of this invention, several types of test kits can be envisioned and produced. First, kits can be made that have a detection device pre-loaded with a detection molecule (or "capture reagent"). In embodiments for detection of IL8Rb mRNA, such devices can comprise a substrate (e.g., glass, silicon, quartz, metal, etc) on which oligonucleotides as capture reagents that hybridize with the mRNA to be detected is bound. In some embodiments, direct detection of mRNA can be accomplished by hybridizing mRNA (labeled with cy3, cy5, radiolabel or other label) to the oligonucleotides on the substrate. In other embodiments, detection of mRNA can be accomplished by first making complementary DNA (cDNA) to the desired mRNA. Then, labeled cDNA can be hybridized to the oligonucleotides on the substrate and detected.

Antibodies can also be used in kits as capture reagents. In some embodiments, a substrate (e.g., a multiwell plate) can have a specific IL8Rb and BTM capture reagents attached thereto. In some embodiments, a kit can have a blocking reagent included. Blocking reagents can be used to reduce non-specific binding. For example, non-specific oligonucleotide binding can be reduced using excess DNA from any convenient source that does not contain IL8Rb and BTM oligonucleotides, such as salmon sperm DNA. Non-specific antibody binding can be reduced using an excess of a blocking protein such as serum albumin. It can be appreciated that numerous methods for detecting oligonucleotides and proteins are known in the art, and any strategy that can specifically detect marker associated molecules can be used and be considered within the scope of this invention.

Antibodies can also be used when bound to a solid support, for example using an antibody chip, which would allow for the detection of multiple markers with a single chip.

In addition to a substrate, a test kit can comprise capture reagents (such as probes), washing solutions (e.g., SSC, other salts, buffers, detergents and the like), as well as

detection moieties (e.g., cy3, cy5, radiolabels, and the like). Kits can also include instructions for use and a package.

Detection of IL8Rb and BTMs in a sample can be preformed using any suitable technique, and can include, but are not limited to, oligonucleotide probes, qPCR or antibodies raised against cancer markers.

It will be appreciated that the sample to be tested is not restricted to a sample of the tissue suspected of being an inflammatory disease or tumour. The marker may be secreted into the serum or other body fluid. Therefore, a sample can include any bodily sample, and includes biopsies, blood, serum, peritoneal washes, cerebrospinal fluid, urine and stool samples.

It will also be appreciate that the present invention is not restricted to the detection of cancer in humans, but is suitable for the detection of cancer in any animal, including, but not limited to dogs, cats, horses, cattle, sheep, deer, pigs and any other animal known to get cancer.

General Tests for inflammatory disease or Cancer Markers in Body Fluids

In general, methods for assaying for oligonucleotides, proteins and peptides in these fluids are known in the art. Detection of oligonucleotides can be carried out using hybridization methods such as Northern blots, Southern blots or microarray methods, or qPCR. Methods for detecting proteins include such as enzyme linked immunosorbent assays (ELISA), protein chips having antibodies, suspension beads radioimmunoassay (RIA), Western blotting and lectin binding. However, for purposes of illustration, fluid levels of a disease markers can be quantified using a sandwich-type enzyme-linked immunosorbent assay (ELISA). For plasma assays, a 5 uL aliquot of a properly diluted sample or serially diluted standard marker and 75 uL of peroxidaseconjugated anti-human marker antibody are added to wells of a microtiter plate. After a 30 minute incubation period at 30°C, the wells are washed with 0.05% Tween 20 in phosphate-buffered saline (PBS) to remove unbound antibody. Bound complexes of marker and anti- marker antibody are then incubated with o-phenyldiamine containing H₂O₂ for 15 minutes at 30°C. The reaction is stopped by adding 1 M H₂SO₄, and the absorbance at 492 nm is measured with a microtiter plate reader.

It can be appreciated that anti- IL8Rb antibodies can be monoclonal antibodies or polyclonal antisera. It can also be appreciated that any other body fluid can be suitably studied.

It is not necessary for a marker to be secreted, in a physiological sense, to be useful. Rather, any mechanism by which a marker protein or gene enters the serum can be effective in producing a detectable, quantifiable level of the marker. Thus, normal secretion of soluble proteins from cells, sloughing of membrane proteins from plasma membranes, secretion of alternatively spliced forms of mRNA or proteins expressed therefrom, cell death (either apoptotic) can produce sufficient levels of the marker to be useful.

There is increasing support for the use of serum markers as tools to diagnose and/or evaluate efficacy of therapy for a variety of cancer types.

Yoshikawa et al., (Cancer Letters, 151: 81-86 (2000) describes tissue inhibitor of matrix metalloproteinase-1 in plasma of patients with gastric cancer.

Rudland et al., (Cancer Research 62: 3417-3427 (2002) describes osteopontin as a metastasis associated protein in human breast cancer.

Buckhaults et al., (Cancer Research 61:6996-7001 (2002) describes certain secreted and cell surface genes expressed in colorectal tumours.

Kim et al., (JAMA 287(13):1671-1679 (2002) describes osteopontin as a potential diagnostic biomarker for ovarian cancer.

Hotte et al., (AJ. American Cancer Society 95(3):507-512 (2002) describes plasma osteopontin as a protein detectable in human body fluids and is associated with certain malignancies.

Martin et al., (Prostate Cancer Prostatic Dis. March 9, 2004 (PMID: 15007379) (Abstract) described use of human kallikrein 2, prostate-specific antigen (PSA) and free PSA as markers for detection of prostate cancer.

Hall et al (Laryngoscope 113(1):77-81 (2003) (PMID: 12679418) (Abstract) described predictive value of serum thyroglobulin in thyroid cancer.

Mazzaferri et al., (J. Clin. Endocrinol. Metab. 88(4):1433-1441 (2003) (Abstract) describes thyroglobulin as a potential monitoring method for patients with thyroid carcinoma.

Whitley et al, (Dim Lab. Med. 24(1):29-47 (2004) (Abstract) describes thyroglobulin as a serum marker for thyroid carcinoma.

Kuo et al (Clin. Chim. Acta. 294(1-2):157-168 (2000) (Abstract) describes serum matrix metalloproteinase-2 and -9 in HCV- and HBV-infected patients.

Koopman et al., (Cancer Epidemiol. Biomarkers Prev 13(3):487-491 (2004) (Abstract) describes osteopontin as a biomarker for pancreatic adenocarcinoma.

Pellegrini et al., (Cancer Immunol. Immunother. 49(7):388-394 (2000) (Abstract) describes measurement of soluble carcinoembryonic antigen and TIMP 1 as markers for pre-invasive colorectal cancer.

Melle et al., (Clin. Chem. 53(4), 629-635 (2007) (Abstract) describes HSP27 as a serum marker for pancreatic adenocarcinoma.

Leman et al., (Urology, 69(4) 714-20 (2007) (Abstract) describes EPCA-2 as a serum marker for prostate cancer.

Tsigkou et al., (J Clin Endocrinol Metab, 92(7) 2526-31 (2007) (Abstract) describes total inhibin as a potential serum marker for ovarian cancer.

Marchi et al., (Cancer 112, 1313-1324 (2008) (Abstract) describes ProApolipoprotein AI as a serum marker of brain metastases in lung cancer patients.

EXAMPLES

The examples described herein are for purposes of illustrating embodiments of the invention. Other embodiments, methods and types of analyses are within the scope of persons of ordinary skill in the molecular diagnostic arts and need not be described in detail hereon. Other embodiments within the scope of the art are considered to be part of this invention.

Example 1:

Methods

Patients: Between April 2008 and September 2009, 471 patients presenting with macroscopic haematuria, but no prior history of urinary tract malignancy, were recruited at eleven urology clinics in New Zealand and Australia. Each patient provided a urine sample immediately prior to undergoing cystoscopy and any additional diagnostic procedures. A diagnosis was made by three months following enrollment in the study. Of these 471 patients, gene expression data on all five study genes was successfully obtained for 442 patients using the methods described below. The characteristics of these patients are shown in Table 4.

Diagnosis	Number
Benign prostatic hyperplasia	18
Cystitis	18
Exercise-induced hematuria	3
Non-specific kidney disease	3
Non-specific neoplasia	3
Non-specific prostate disease	63
Vascular prostate	49
Other urological cancer (non-TCC)	5
Superficial vessels	3
Urethral stricture	6
Urinary tract infection	18
Urolithiasis	25
Warfarin use	10
Unknown etiology	155
Miscellaneous	7
TCC	56
Total	442

Table 4. Characteristics of the study population. The table shows the number of patients in each of the main diagnostic categories at three months after the patient's initial presentation with gross hematuria.

Urine analysis: Urine samples were analysed by central review cytology (Southern Community Laboratories, Dunedin, New Zealand). The diagnostic tests NMP22 BladderChek (Matritech) and NMP22 ELISA (Matritech) were carried out according to the manufacturer's instructions at the clinical site (BladderChek) or by Southern Community Laboratories (NMP22 ELISA).

RNA quantification: 2mls of urine from each patient was mixed with RNA extraction buffer containing 5.64M guanidine thiocyanate, 0.5% sarkosyl and 50mM NaoAc pH6.5. Total RNA was then extracted by Trizol extraction (Invitrogen) and the RNeasy procedure (Qiagen), as previously described¹. RNA was eluted from the columns in 35ul water and 3ul was used in each subsequent monoplex or duplex quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. Each 16ul qRT-PCR reaction contained 0.3U RNase-OUT (Invitrogen), 0.225uM each Taqman probe, 1.25U Superscript III (Invitrogen), 0.275uM each primer, 1.5U Fast Start Taq polymerase (Roche), 10mM DTT, 0.375mM dNTPs, 4.5mM MgSO₄, 1.6ul 10x Fast Start PCR buffer (Roche) and 2.6ul GC Rich solution (Roche). Primers and fluorescently dual-labeled probes were obtained from Integrated DNA

Technologies (Coralville USA) for each of the five study genes: MDK, CDC2, HOXA13, IGFBP5 and IL8Rb. Primer/probe sequences are shown in Table 2. Reactions were set up in 96 well plates and cycled as follows on a Roche Light Cycler® 480: 50°C, 15 mins; 95°C 8 mins; 10 cycles of 95°C 15 sec, 60°C 2 mins and 30 cycles of 95°C 15 secs, 60°C 1 min. Standard curves of 1/16 serial dilutions of a reference RNA (derived from pooled cell line RNAs) were included on each plate to generate range of 0.3 pg/μl to 20 ng/μl. Data was collected at the extension phase of the final 30 thermocycles and exported as a raw text file.

Marker	Forward Seq	Reverse Seq	Probe
MDK	TGC ACC CCC AAG ACC AAA (Seq ID No 3)	TGA TTA AAG CTA ACG AGC AGA CAG AA (Seq ID No 4)	CCT TCC CTT TCT TGG CTT TGG CCT TT (Seq ID No 5)
IGFBP5	CGT TGT ACC TGC CCA ATT GTG A (Seq ID No 6)	GGG ACG CAT CAC TCA ACG TT (Seq ID No 7)	AAG AGA AAG CAG TGC AAA CCT TCC CGT (Seq ID No 8)
CDC2	GCC GCC GCG GAA TAA T (Seq ID No 9)	TGT CTA CCC TTA TAC ACA ACT CCA TAG G (Seq ID No 10)	AGC CGG GAT CTA CCA TAC CCA TTG ACT AAC T (Seq ID No 11)
HOXA13	TGG AAC GGC CAA ATG TAC TG (Seq ID No 12)	TGG CGT ATT CCC GTT CAA GT (Seq ID No 13)	ACT CTG CCC GAC GTG GTC TCC CA (Seq ID No 14)
IL8Rb	CCT TGA GGC ACA GTG AAG ACA TC (Seq ID No 15)	CCT GTA GGA CAC CTC CAG AAG AG (Seq ID No 16)	TGG CCA CTC CAA TAA CAG CAG GTC ACA (Seq ID No 17)

Table 5: Primer and probe sequences used for the qRT-PCR quantification of the five RNA markers.

qRT-PCR data analysis: Raw fluorescence data was exported from the Roche LightCycler® 480 as a tab-delimited file containing cycle number versus two channels of fluorescence data for all wells on the plate. The data were processed using an R program that applied colour compensation ([Bernard1999]) to the data to correct for bleed over from one fluorescent channel into another. It then fitted a 5-point logistic model to estimate the C_p using the second derivative maximum ([Spiess2008]).

All samples and controls were applied in duplicate to the PCR plates. The C_p values from the duplicate wells were averaged before use. If the difference between the two C_p values exceeded 3 units, that sample was repeated. To provide standardization across PCR plates, C_p 's were expressed as ΔC_p 's relative to a reference RNA (derived from pooled cell line

RNAs) at 20 ng/μl:

$$\Delta C_P = C_P (\text{sample}) - C_P (\text{reference RNA})$$

Statistical Analysis: qRT-PCR ΔC_P values from MDK, CDC2, HOXA13, IGFBP5 and IL8Rb were used to generate classifiers to separate samples containing TCCs from samples containing no TCCs, based on Linear Discriminant Analysis or Logistic Regression ([Venables2002]). In both cases, interactions between genes were permitted in the classifier models. The generation of the LDA followed standard procedures, as described, for example in "Modern Applied Statistics with S, 4th edition" by W.N. Venables and B.D. Ripley (2002), Springer. The dataset from the study was cleaned of any incomplete data then the R Statistical Environment (R Development Core Team (2009) and the function "lda" from the package MASS (Venables and Ripley (2002)) were used to generate and test the linear discriminant on the clinical trial data.

The generation of the Logistic Regression classifier was performed in a similar manner to the generation of the LDA. Again, the study data was cleaned of incomplete data. A logistic regression classifier was created using R; no additional packages were required. Logistic regression was performed as described by Dalgaard (2008). Comparison among classifiers was made using ROC curves, using the R package, ROCR (Sing et al. 2009). Confidence intervals for ROC curves were generated using the methods of Macskassy et al ([Macskassy2005]).

The following algorithms were generated:

Linear Discriminant Classifier

The first classifier, a linear discriminant, (called LDA-3), is based on five gene values (normalized to a Reference value by subtracting the reference value) allowing for multiway interactions between the genes. The classifier was built in R using the 'lda()' function from the package called "MASS". (R version 2.9.1; MASS version 7.2-49). The classifier was built using the following equation:

```
lda3 <- lda(TCC.YN ~ MDK * IGF * CDC * HOXA * IL8R, data=uRNA.Trial)
```

Where `lda3` is the created model; `TCC.YN` is the true value for “presence of TCC in urine” as determined by our gold-standard, cystoscopy; `MDK`, `IGF`, `CDC`, `HOXA` and `IL8R` are the normalized gene Cp value; and `uRNA.Trial` is a data frame contain the values for the genes and `TCC.YN` from the clinical trial. Use of the ASTERISK, “*”, in the formula does not signify multiplication, but rather means “interacting terms” in the classifier.

Evaluation of the classifier score takes as input a new data frame containing the five gene values as well as the classifier, `lda3`, to output a classifier score:

```
score <- c(predict(lda3, new.data)$x)
```

Where `score` is the output used from the classifier to predict the presence of TCCs; `lda3` is the classifier created above and `new.data` is a data frame containing the measured values of the five genes called by the same names as used in classifier creation. The syntax, ‘`$x`’ and `c( )`, is present to extract the score specifically from the large amount of information returned by the `predict` function.

Setting the score cut off to 0.112 and above, sets our specificity to 85% for presence of TCCs in the urine sample.

The coefficients for LDA-3 are:

<code>MDK.d.R100</code>	
<code>5.333639e+00</code>	
<code>IGF.d.R100</code>	
<code>3.905978e+00</code>	
<code>CDC.d.R100</code>	<code>6.877143e-</code>
<code>01</code>	
<code>HOXA.d.R100</code>	
<code>6.073742e+00</code>	
<code>IL8R.d.R100</code>	<code>-</code>
<code>1.229466e+00</code>	
<code>MDK.d.R100:IGF.d.R100</code>	<code>-7.420480e-</code>
<code>01</code>	
<code>MDK.d.R100:CDC.d.R100</code>	<code>-2.611158e-</code>
<code>01</code>	
<code>IGF.d.R100:CDC.d.R100</code>	<code>-1.965410e-</code>
<code>01</code>	
<code>MDK.d.R100:HOXA.d.R100</code>	<code>-8.491556e-</code>
<code>01</code>	
<code>IGF.d.R100:HOXA.d.R100</code>	<code>-4.037102e-</code>
<code>01</code>	
<code>CDC.d.R100:HOXA.d.R100</code>	<code>-3.429627e-</code>
<code>01</code>	

MDK.d.R100:IL8R.d.R100 01	1.903118e-
IGF.d.R100:IL8R.d.R100 01	2.684005e-
CDC.d.R100:IL8R.d.R100 01	-1.229809e-
HOXA.d.R100:IL8R.d.R100 01	2.909062e-
MDK.d.R100:IGF.d.R100:CDC.d.R100 02	4.108895e-
MDK.d.R100:IGF.d.R100:HOXA.d.R100 02	7.664999e-
MDK.d.R100:CDC.d.R100:HOXA.d.R100 02	4.832034e-
IGF.d.R100:CDC.d.R100:HOXA.d.R100 02	2.116340e-
MDK.d.R100:IGF.d.R100:IL8R.d.R100 02	-3.750854e-
MDK.d.R100:CDC.d.R100:IL8R.d.R100 02	1.664612e-
IGF.d.R100:CDC.d.R100:IL8R.d.R100 03	2.089442e-
MDK.d.R100:HOXA.d.R100:IL8R.d.R100 02	-1.539486e-
IGF.d.R100:HOXA.d.R100:IL8R.d.R100 02	-3.894153e-
CDC.d.R100:HOXA.d.R100:IL8R.d.R100 03	6.295032e-
MDK.d.R100:IGF.d.R100:CDC.d.R100:HOXA.d.R100 03	-4.359738e-
MDK.d.R100:IGF.d.R100:CDC.d.R100:IL8R.d.R100 04	-2.019317e-
MDK.d.R100:IGF.d.R100:HOXA.d.R100:IL8R.d.R100 03	3.746882e-
MDK.d.R100:CDC.d.R100:HOXA.d.R100:IL8R.d.R100 03	-2.902150e-
IGF.d.R100:CDC.d.R100:HOXA.d.R100:IL8R.d.R100 04	4.799489e-
MDK.d.R100:IGF.d.R100:CDC.d.R100:HOXA.d.R100:IL8R.d.R100 05	7.512308e-

Logistic Regression Classifier

A second classifier based on Logistic Regression was derived from the same cleaned dataset as LDA-3. Instead of using the `lda()` function, however, we used the `glm()` function from the package `stats` (included with a base install of R) as shown below:

```
lr1 <- glm(TCC.YN ~ CDC * IGF * HOXA * IL8R * MDK,
           family=binomial("logit"), data=uRNA.Trial )
```

Where `lr1` is the classifier created and the other parameters are as described for the linear discriminant. Once again, full interaction is specified using the `'**'` operator. Classification is performed in a manner very similar to that for LDA-3:

```
score <- predict(lr1, new.data, type='response')
```

Where `score` is the value used to classify urine samples based on the measurement of the five genes in `new.data`, as above. The cut off for `lr1` is set to 0.102 to achieve a specificity of 85%; values about the cut off are considered to be positive to TCCs. The coefficients for the classifier are:

```
-103.0818143 +
3.9043769 * CDC.d.R100 +
13.1120675 * IGF.d.R100 +
17.4771819 * HOXA.d.R100 +
-10.7711519 * IL8R.d.R100 +
21.1027595 * MDK.d.R100 +
-0.5938881 * CDC.d.R100 * IGF.d.R100 +
-1.0736184 * CDC.d.R100 * HOXA.d.R100 +
-1.3340189 * IGF.d.R100 * HOXA.d.R100 +
0.3126461 * CDC.d.R100 * IL8R.d.R100 +
1.4597355 * IGF.d.R100 * IL8R.d.R100 +
1.8739459 * HOXA.d.R100 * IL8R.d.R100 +
-1.035054 * CDC.d.R100 * MDK.d.R100 +
-2.5885156 * IGF.d.R100 * MDK.d.R100 +
-2.7013483 * HOXA.d.R100 * MDK.d.R100 +
1.4546134 * IL8R.d.R100 * MDK.d.R100 +
0.0767503 * CDC.d.R100 * IGF.d.R100 * HOXA.d.R100 +
-0.0663361 * CDC.d.R100 * IGF.d.R100 * IL8R.d.R100 +
-0.1015552 * CDC.d.R100 * HOXA.d.R100 * IL8R.d.R100 +
-0.2110656 * IGF.d.R100 * HOXA.d.R100 * IL8R.d.R100 +
0.1361215 * CDC.d.R100 * IGF.d.R100 * MDK.d.R100 +
0.1601118 * CDC.d.R100 * HOXA.d.R100 * MDK.d.R100 +
0.259745 * IGF.d.R100 * HOXA.d.R100 * MDK.d.R100 +
-0.0106468 * CDC.d.R100 * IL8R.d.R100 * MDK.d.R100 +
-0.1947899 * IGF.d.R100 * IL8R.d.R100 * MDK.d.R100 +
-0.185286 * HOXA.d.R100 * IL8R.d.R100 * MDK.d.R100 +
0.0136603 * CDC.d.R100 * IGF.d.R100 * HOXA.d.R100 * IL8R.d.R100 +
-0.0151368 * CDC.d.R100 * IGF.d.R100 * HOXA.d.R100 * MDK.d.R100 +
0.0056651 * CDC.d.R100 * IGF.d.R100 * IL8R.d.R100 * MDK.d.R100 +
0.0030538 * CDC.d.R100 * HOXA.d.R100 * IL8R.d.R100 * MDK.d.R100 +
0.0232556 * IGF.d.R100 * HOXA.d.R100 * IL8R.d.R100 * MDK.d.R100 +
-0.000867 * CDC.d.R100 * IGF.d.R100 * HOXA.d.R100 * IL8R.d.R100 *
MDK.d.R100
```

Results

qRT-PCR analysis of urine samples

To obtain an overview of the effect of IL8Rb on TCC detection, two dimensional scatter plots were constructed using qRT-PCR data obtained from the urine of patients with either TCC (n= 56) or the non-malignant conditions urolithiasis (n=25), urinary tract infection (n=18) or cystitis (n=18). The scatter plots were constructed using pairs of genes from a four gene signature (MDK, CDC2, HOXA13, IGFBP5). IL8Rb was then substituted for one gene of each pair and the data re-plotted. These plots are shown in Figure 2a-f. Substitution of IL8Rb for IGFBP5 and HOXA13 in plots with MDK (Figure. 2a-c) showed improved separation between samples from patients with TCC and those with non-malignant conditions. The same trend was observed in plots with CDC2 in which IL8Rb was substituted for IGFBP5 and HOXA13 (Figure. 2d-f).

The contribution of IL8Rb to the correct diagnosis of TCC in patients presenting with gross hematuria was then quantified by ROC curve analysis. qRT-PCR data for each gene in the signature (MDK, CDC2, IGFBP5 and HOXA13) and IL8Rb was used to develop linear discriminate algorithms that maximised the discrimination between the patients with TCC and those without. Two linear discriminate algorithms were developed using the entire cohort of 442 samples: LD1, which used the qRT-PCR data from MDK, CDC2, HOXA13 and IGFBP5 and LD2, which used MDK, CDC2, HOXA13, IGFBP5 and IL8Rb. LD1 and LD2 were then used to generate ROC curves showing the sensitivity and specificity of TCC detection in the group of patients with confirmed TCC (n=56) or the non-malignant conditions urolithiasis (n=25), urinary tract infection (n=18) or cystitis (n=18). Figure 3a shows the ROC curves for LD1 and LD2. The area under the ROC curve for LD1 was 78% compared to 84% for LD2.

As an alternative to linear discriminate analysis, logistic regression was used as an independent method to develop an algorithm for the discrimination between patients with TCC and those with non-malignant disease. As for the linear discriminate analysis, the logistic regression algorithms were developed using the entire cohort of 442 samples. The ROC curves obtained using logistic regression and the 56 TCC and 61 non-malignant samples described above are shown in Figure 3b. The area under the ROC curve for LR1 (obtained using qRT-PCR data from MDK, CDC2, HoxA13 and IGFBP5) was 80% compared to 86% for LR2 (obtained using qRT-PCR data from MDK, CDC2, HOXA13, IGFBP5 and IL8Rb). This data clearly illustrates that inclusion of IL8Rb in methods for the detection of TCC using urine samples can lead to improved discrimination between patients with TCC and non-malignant diseases such as cystitis, urinary tract infection and urolithiasis.

To confirm the improved accuracy afforded by IL8Rb for the discrimination between patients with TCC and urolithiasis, urinary tract infection or cystitis was maintained in an unselected cohort of patients comprising a larger number and diversity of non-malignant patients, the ROC curve analyses were repeated with the entire cohort of 442 samples described in Table 1. In this analysis, the area under the curve for LD1 and LD2 was 86 and 89%, respectively (Figure. 4a). Similarly, the area under the curve for LR1 was 87% and for LR2 91% (Figure. 4b). This result confirms that IL8Rb leads to improved accuracy in the detection of TCC using urine samples.

This improvement in cancer detection due to the inclusion of IL8Rb was further illustrated by applying LD1/LD2 and LR1/LR2 to the 442 patient cohort and then determining the sensitivity of detection of stage Ta TCC alone. Stage Ta tumours are smaller, more differentiated tumours that are typically more difficult to detect than higher stage tumours. LD1 detected 18/31 (58%) of the Ta tumours compared to 19/31 (61%) for LD2 at a specificity of 85%. LR1 detected 21/31 (68%) compared to 24/31 (77%) for LR2 (specificity of 85%). This data shows that the inclusion of IL8Rb into the LD and LR algorithms increased the sensitivity of detection of stage Ta tumours by up to 9%. In comparison to these RNA tests, the three other bladder cancer tests in this study showed markedly lower accuracy for the detection of Ta tumours: urine cytology (39% sensitivity, 94% specificity), NMP22 ELISA (35% sensitivity, 88% specificity) and NMP22 (BladderChek® “a registered trademark of Matritech, Inc. of Massachusetts, United States”) (39% sensitivity, 96% specificity).

IL8Rb as an aid in the diagnosis of inflammation of the urinary tract.

To determine the ability of IL8Rb to be used in the diagnosis of patients with inflammation of the urinary tract due to causes such as cystitis or urinary tract infections, the urine levels of IL8Rb mRNA in haematuria patients diagnosed with benign prostate hyperplasia, non-specific prostate disease, vascular prostate, hematuria secondary to warfarin use, and cystitis/urinary tract infection were determined by qRT-PCR. The mean IL8Rb ΔC_t levels for each of these conditions were -3.12, -3.10, -2.84, -1.98 and -5.27, respectively. The difference between the mean of the IL8Rb level in patients with cystitis/urinary tract infection and the other non-malignant states combined was determined to be significant ($p=0.001$) using the Wilcoxon rank sum test. Box plots portraying this data are shown in figure 5. This data shows an elevation of IL8Rb levels in the majority of patients diagnosed with either cystitis or urinary tract infection compared to the other non-malignant conditions examined. Overlap between plots is likely to be explained by a combination of three factors: (i) the inability of standard clinical practice to correctly diagnose each condition, (ii) co-morbidity (eg

infection and benign prostate hyperplasia), and (iii) the normal association of high urine neutrophil counts in a subset of patients with benign prostate hyperplasia, non-specific prostate disease, vascular prostate or hematuria secondary to warfarin use. Regardless, given the strict association between inflammation and neutrophil numbers, the quantification of IL8Rb in urine provides an accurate method of detecting inflammation of the urinary tract, be it as a consequence of infection or in association with other non-malignant conditions.

Example 2

Methods

Study population

A consecutive series of patients without a prior history of TCC were recruited prospectively from nine urology clinics in New Zealand and two in Australia between 28th April 2008 and 11th August 2009. The patient set included the patients used in example 1, but included an additional 46 patients, whose data was not available for the first analysis. The further studying also includes further analysis of the results obtained.

The samples were collected and RNA collected and tested as described in Example 1.

RNA test development

uRNA[®] consists of four mRNA markers, *CDC2*, *HOXA13*, *MDK* and *IGFBP5*. These markers were selected on the basis of their low expression in blood and inflammatory cells and over-expression in TCC.² In this cohort study, we prospectively specified a linear discriminate algorithm (uRNA-D) that combined the four markers into a single score. uRNA-D was independent, being developed on an earlier dataset. It was not however, derived using a strictly characterised patient group representing the intended target population for the test. As a consequence, the study protocol also defined the development of a new algorithm (*Classifier-D*) for the use of the five markers *CDC2*, *HOXA13*, *MDK*, *IGFBP5* and *IL8Rb* using data obtained from the patients recruited to the current cohort study.

In addition to *Classifier-D*, a second algorithm (*Classifier-S*) was derived using the cohort study data to enable identification of tumours that were either of advancing stage ($\geq$ stage 1) or high grade (WHO/ISUP 1998 classification). Algorithm-S comprised all five markers, including *CDC2* and *HOXA13* which had previously been shown to be differentially expressed between Stage Ta tumours and those $\geq$ stage 1.

Classifier development

Development of two classifiers for the use of the five markers *CDC2*, *HOXA13*, *MDK*, *IGFBP5* and *IL8Rb* (*Classifier-D* and *Classifier-S*) were based on data obtained in this study, in accordance with the methods outlined in this specification. Briefly, logistic regression models were made using the statistical programming environment, R (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL [http:// www.R-project.org/](http://www.R-project.org/)). Models made using ΔC_p values for each of the five markers and their two way interactions (eg. *MDK* x *CDC2*, *MDK* x *IGFBP5*, etc) were evaluated for their ability to classify; those with the lowest AIC values were evaluated in a leave-one-out cross validation procedure for their sensitivity when the specificity was set to 85%. Several models demonstrated comparable performance for each of *Classifier-D* and *Classifier-S*, with the model with the fewest numbers of parameters being selected.

Statistical methods

Where a diagnostic test was specified in the protocol, proportions and 95% confidence intervals were calculated for sensitivity and specificity. Received operating characteristic (ROC) curves were plotted and compared using the Stata *roctab* and *roccomp* commands (Statacorp and Delong). For *Classifier-D* confidence intervals are not appropriate, but Fishers exact or Chi squared tests (where sample sizes allow) were used to test for an association between TCC or patient characteristics and chances of true positive or false positive results. Logistic regression models were used to explore factors associated with false positive and false negative results. All analyses were carried out in Stata version 11.2.

RESULTS

A total of 517 patients were initially recruited to the study; 4% of patients were excluded because they were found to be ineligible (n=10), did not undergo cystoscopy (n=9), TCC status was not stated (n=2) or they did not provide an acceptable urine sample (n=2) (Figure 8). A further 10 patients were excluded from the analysis because they did not have results for one or more of the urine tests. The baseline demographic and clinical characteristics of the 485 remaining patients are shown in Figure 9.

The prevalence of TCC in the cohort was 13.6%. Two were missing a review stage (both were Ta by local review) and two were not given a review grade (one was grade 1 by the local pathologist, the other low). Of the 66 tumours, 55 were superficial (stage Ta, T1 or Tis)

and 11 were muscle invasive (T2). No patients had detectable metastases or involvement of regional lymph nodes. Using the 1973 grading system, 24 were classified as grade 3, 38 grade 2, three grade 1 and one unknown. With the WHO98 system, 29 were classified as high grade, four mixed, 32 low grade and one unknown. In addition to the TCCs, two patients were diagnosed with a papilloma, and seven with other neoplasms (five of these urological).

The cutoff for the uRNA-D test was determined on the study cohort, with specificity set at 85%. With this cutoff, uRNA-D detected 41 of the 66 TCC cases (sensitivity of 62%), compared with NMP22™ ELISA (50%), Bladderchek™ (38%) and cytology (56%). The RNA test developed on the cohort data Classifier-D detected 54 of the TCC cases (82%) at a specificity of 85% and 48 (73%) at a specificity of 90%. uRNA-D and NMP22™ ELISA values can be directly compared as both tests were fully specified prior to the study. Figure 2a shows the ROC curves; the area under the curves (AUCs) are 0.81 and 0.73 respectively ($p=0.03$). The ROC curve for Classifier-D was 0.87 (Figure 11b), and the improvement in performance relative to uRNA-D appears to be mostly in the range of clinically relevant specificities (above 80%).

Overall, Classifier-D detected 97% of the high/grade 3 tumours, compared to uRNA-D (83%), cytology (83%), NMP22 ELISA (69%) and Bladderchek (38%). Classifier-D was also more sensitive for the detection of low grade tumours (69%), with the other tests ranging from 28-41% (Figure 12). Classifier-D was positive for all the TCC cases of Stage ≥ 1 plus both Tis, but the sensitivity was 68% for stage Ta ($p=0.016$, Figure 12). This was still substantially higher than the other tests, with uRNA-D being the next highest at 41%. TCC patients with microhaematuria evident in their urine sample were more likely to have their TCC detected by including IL8Rb than those without microhaematuria ($p<0.0005$), though this is likely to be at least partially a result of the higher proportion of high stage and grade TCCs among those with microhaematuria. Numbers were insufficient to explore this further in regression analyses.

Of the 12 cases missed by Classifier-D, all were stage Ta and all except one were low grade (WHO ISUP 1998). Only two of the twelve (both low grade, stage Ta TCC) were picked up by another test (one by both NMP22™ ELISA and BladderChek™ and one by uRNA-D). Of the 12 cases missed by Classifier-D, all were stage Ta and all except one were low grade (WHO ISUP 1998). Only two of the twelve (both low grade, stage Ta TCC) were picked up by another test (one by both NMP22™ ELISA and BladderChek™ and one by uRNA-D). Cytology did not pick up any TCCs that Classifier-D missed.

Patient A : High Grade renal pelvic T2 tumour, no concurrent Tis, no size given.

Patient B: High grade Bladder T3a no concurrent Tis, 2x 3cm

Patient C : a high grade tumour measuring 4.8 x 5.6 cm with extensive stromal and muscularis propria invasion, extending to the perivesical fat with no evidence of metastasis.

The specificity of the urine tests among those with alternative diagnoses and according to urine sample characteristics are shown in Figure 13. Control patients with microhaematuria were more likely to have false positive tests than those without microhaematuria ($p=0.002$), and there was a suggestion that patients with calculi may as well, although the differences in specificity by diagnosis were not statistically significant overall ($p=0.12$). There were five patients with other urological cancers; only one of these gave a positive Classifier-D test result. Results from fitting logistic regression models were similar. In a logistic regression model with diagnosis and microhaematuria, the association with microhaematuria status remained significant ($p=0.006$) and, when compared directly to no diagnosis those with calculi had a 2.7 fold increased odds of a false positive test (95% CI (1.1 to 6.4), $p=0.03$). Age did not affect the specificity of the test.

Microhaematuria detected in the urine sample was the only factor clearly associated with test sensitivity. The predictive value of a positive test in this cohort was 63% for those with microhaematuria and 24% for those without, largely reflecting the greater prevalence of TCC in the patients with microhaematuria (39% vs 6%).

There were 54 patients with TCC in whom the Classifier-D test was positive. These patients were classified into severe and less severe TCC using Classifier-S. Severe TCC was defined as stage ≥ 1 or grade 3 at any stage. At a specificity of 90%, Classifier-S correctly classified 32/35 (91%) of the severe TCC cases.

References

Altekruse SF, Kosary CL, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlander N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK (eds). *SEER Cancer Statistics Review, 1975-2007*, National Cancer Institute. Bethesda, MD, [http:// seer.cancer.gov/csr/1975_2007/](http://seer.cancer.gov/csr/1975_2007/), based on November 2009 SEER data submission, posted to the SEER web site, 2010.

Byrd, R. H., Lu, P., Nocedal, J. and Zhu, C. (1995) A limited memory algorithm for bound constrained optimization. *SIAM J. Scientific Computing*, 16, 1190–1208.

Dalgaard (2008). "Introductory Statistics with R, 2nd edition" ; Peter Dalgaard, Chapter 13 (2008), Springer, ISBN 978-0-387-79053-4.

DeLong, E. R., D. M. DeLong, and D. L. Clarke-Pearson. 1988. Comparing the areas under two or more correlated receiver operating characteristic curves: A nonparametric approach. *Biometrics* 44: 837-845.

Gottschalk, P.G. and Dunn, J.R. (2005) The five-parameter logistic: A characterization and comparison with the four-parameter logistic. *Analytic Biochemistry*, 343, 54-65.

Doi:10.1016/j.ab.2005.04.035

Hoerl, A.E. (1962) Application of ridge analysis to regression problems. *Chemical Engineering Progress*, 58, 54-59.

Holyoake A, O'Sullivan P, Pollock R, Best T, Watanabe J, Kajita Y, et al. Development of a multiplex RNA urine test for the detection and stratification of transitional cell carcinoma of the bladder. *Clin Cancer Res*. 2008 Feb 1;14(3):742-9.

Lunn, D., Spiegelhalter, D., Thomas, A. and Best, N. (2009) The BUGS project: Evolution, critique and future directions (with discussion), *Statistics in Medicine* 28: 3049--3082.

Moré, J.J. (1978) The Levenberg-Marquardt algorithm: implementation and theory, in *Lecture Notes in Mathematics* 630: Numerical Analysis, G.A. Watson (Ed.), Springer-Verlag: Berlin, pp. 105-116.

Nelder, J. A. and Mead, R. (1965) A simplex algorithm for function minimization. *Computer Journal* 7, 308–31.

(R Development Core Team (2009). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>)

Richards, F.J. (1959) A flexible growth function for empirical use. *Journal of Experimental Botany*. 10, 290-300.

Sing et al (2009). (Tobias Sing, Oliver Sander, Niko Beerenwinkel and Thomas Lengauer (2009). ROCR: Visualizing the performance of scoring classifiers.. R package version 1.0-4. <http://CRAN.R-project.org/package=ROCR>).

Speiss, A.-N., Feig, C. and Ritz, C. (2008) Highly accurate sigmoidal fitting of real-time RCR data by introducing a parameter for asymmetry. *BMC Bioinformatics*, 9, 221. Doi:10.1186/1471-2105-9-211.

Sprenger H, Lloyd AR, Lautens LL, Bonner TI, Kelvin DJ. Structure, genomic organization, and expression of the human interleukin-8 receptor B gene. *J Biol Chem*. 1994 Apr 15;269(15):11065-72.

StataCorp. 2009. *Stata Statistical Software: Release 11*. College Station, TX: StataCorp LP.

Venables, W. N. & Ripley, B. D. (2002). *Modern Applied Statistics with S*. Fourth Edition. Springer, New York. ISBN 0-387-95457-0).

Claims

1. A method for detecting bladder cancer, comprising:
 - (i) providing a urine sample;
 - (ii) detecting the levels of the human neutrophil marker gene interleukin 8 receptor B (IL8Rb) in association with bladder cancer marker (BTM) genes in said sample wherein the BTM genes comprise cell division cycle 2, G1 to S and G2 to M (CDC2), midkine (neurite growth-promoting factor 2) (MDK), Homeo Box A13 (HOXA13), and insulin-like growth factor binding protein 5 (IGFBP5); and
 - (iii) wherein the presence of cancer is established by comparing the levels of IL8Rb and the BTM genes with the levels in normal patients, and/or patients having bladder cancer, and/or patients having an inflammatory bladder condition, and establishing if the patient has bladder cancer,wherein an increase in the levels of the BTM genes, without an increase in the level of IL8Rb, is indicative of the patient having bladder cancer, and wherein an increase in the level of IL8Rb is indicative of the patient having an inflammatory bladder condition.
2. The method of claim 1, wherein:
 - (a) the likelihood of the presence of cancer is determined by establishing a diagnostic threshold based on the known expression levels of IL8Rb and the BTM genes with the levels in normal patients, and/or patients having bladder cancer, and/or patients having an inflammatory bladder condition, optionally wherein the threshold is established using a statistical method and optionally wherein the statistical method is any one of Linear Discriminant Analysis (LDA), Logistic Regression (LogReg), Support Vector Machine (SVM), K-nearest 5 neighbors (KN5N), and Partition Tree Classifier (TREE); and/or
 - (b) the BTM genes includes at least one additional marker gene selected from Figure 6 or Figure 7.
3. The method of any one of the preceding claims, wherein:
 - (a) said step of detecting is carried out by detecting the levels of mRNA; or
 - (b) said step of detecting is carried out by detecting the levels of cDNA, optionally using an oligonucleotide complementary to at least a portion of said cDNA, or using qRT-PCR method using a forward primer and a reverse primer.

4. The use of a device for detecting a bladder cancer in a urine sample, the device comprising:

a substrate having a IL8Rb gene capture reagent and capture reagent for bladder tumour marker (BTM) genes thereon wherein the BTM genes comprise cell division cycle 2, G1 to S and G2 to M (CDC2), midkine (neurite growth-promoting factor 2) (MDK), Homeo Box A13 (HOXA13), and insulin-like growth factor binding protein 5 (IGFBP5); and

a detector associated with said substrate, said detector capable of detecting the expression associated with said capture reagent,

wherein an increase in the levels of the BTM genes, without an increase in the level of IL8Rb, is indicative of the patient having bladder cancer, and wherein an increase in the level of IL8Rb is indicative of the patient having an inflammatory bladder condition.

5. The use of claim 4, wherein the BTM genes includes at least one additional marker selected from Figure 6 or Figure 7.

6. The use of any one of claims 4 to 5, wherein:

- (a) said capture reagent is an oligonucleotide; or
- (b) said capture reagent is an antibody specific for either a oligonucleotide, a protein or a peptide.

7. The use of a kit for detecting bladder cancer in a urine sample, the kit comprising:

a substrate having a IL8Rb gene capture reagent and a capture reagent for bladder tumour marker (BTM) genes thereon wherein the BTM comprise cell division cycle 2, G1 to S and G2 to M (CDC2), midkine (neurite growth-promoting factor 2) (MDK), Homeo Box A13 (HOXA13), and insulin-like growth factor binding protein 5 (IGFBP5);

a means for visualizing a complex of said capture agent and a marker; reagents; and instructions for use,

wherein an increase in the levels of the BTM genes, without an increase in the level of IL8Rb, is indicative of the patient having bladder cancer, and wherein an increase in the level of IL8Rb is indicative of the patient having an inflammatory bladder condition.

8. The use of claim 7, wherein:

- (a) said capture reagent is an oligonucleotide; or
- (b) said capture reagent is an antibody selective for a oligonucleotide, a protein or a peptide.

- 9.** A method for screening for bladder cancer, comprising the steps of:
- providing a test urine sample from a test subject;
 - measuring the presence of the IL8Rb gene and bladder tumour marker (BTM) genes in said test urine sample wherein the BTM genes comprise cell division cycle 2, G1 to S and G2 to M (CDC2), midkine (neurite growth-promoting factor 2) (MDK), Homeo Box A13 (HOXA13), and insulin-like growth factor binding protein 5 (IGFBP5); and
 - comparing the amount of markers present in said test urine sample with a value obtained from a control urine sample from a subject not having bladder cancer, and/or with a control sample with a subject having an inflammatory condition of the bladder and establishing if bladder cancer is present,
- wherein an increase in the levels of the BTM genes, without an increase in the level of IL8Rb, is indicative of the presence of bladder cancer, and wherein an increase in the level of IL8Rb is indicative of the presence of an inflammatory bladder condition.
- 10.** The method of claim 9, wherein:
- (a) said marker detected is an oligonucleotide, optionally DNA or RNA; and/or
 - (b) said step of measuring uses an ELISA assay.