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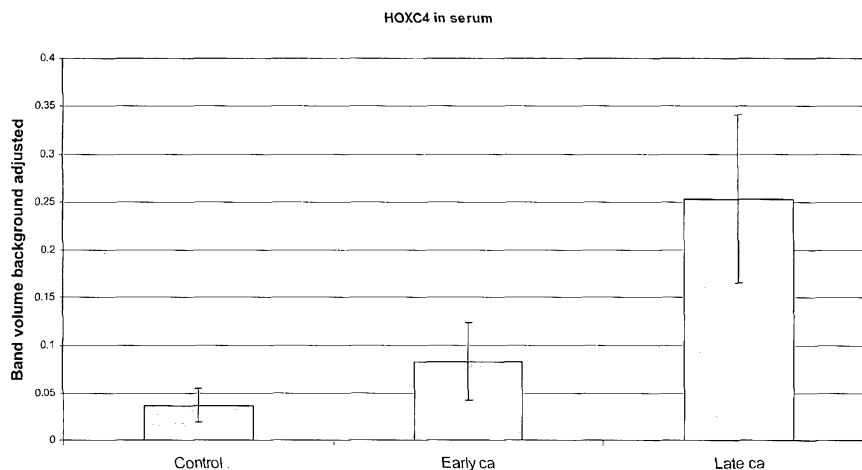
(57) **Abstract:** Provided is a biomarker for detecting cancer, in particular prostate cancer in a male subject, the biomarker comprising at least one homeodomain containing transcription factor, such as a HOX peptide or EN-2 peptide, or a fragment thereof. The use of said biomarker in detecting and/or treating prostate cancer is provided, together with methods and kits therefor.

Cancer Biomarkers

The present invention relates to the use of at least one homeodomain containing transcription factor, such as a HOX peptide or EN-2 peptide, as a biomarker for prostate cancer.

Prostate cancer is currently the leading cause of cancer related death in men, killing 10,000 men each year in the UK alone. However, the current availability of biomarkers for the non-invasive detection of prostate cancer is essentially limited to Prostate Specific Antigen (PSA). Serum levels of PSA are significantly raised in prostate cancer patients, and the PSA test is currently used to follow the progress of the disease and its response to treatment.

Unfortunately, there are a number of significant disadvantages with the PSA test. As PSA levels are also significantly raised in other, non-cancerous conditions of the prostate, such as prostatitis and benign hypertrophy, it is not always certain that detecting an increase in PSA levels is actually indicative of prostate cancer in a patient. Furthermore, PSA levels do not necessarily correlate with the volume of the disease or to the development of nodal or bone metastasis.

Recent attempts to use indicia such as the ratio of free to total PSA and "PSA velocity" proved largely unsuccessful, due to the very high level of false positives, resulting in a large number of unnecessary invasive biopsies.

Indeed, the levels of PSA are thought to rise only very slowly in prostate cancer, so that the disease is often far advanced before a positive result is obtained, when measuring PSA levels. Furthermore, PSA levels have to be normalised, as they can vary with age and prostate size.

There are also a number of additional potential chemical markers of prostate cancer including the kallikreins, and more recently, the 80kDa fragments of e-cadherin (Kuefer *et al.*, 2005). Again, these biomarkers tend to show many of the significant disadvantages discussed above in relation to PSA.

Thus, there is a need in the art for a reliable prostate cancer biomarker.

Surprisingly, we have found that a homeotic transcription factor, HOXC4, is secreted from prostate tumours and its serum concentration is significantly elevated in patients of prostate cancer. Thus, the presence of HOXC4 in body fluids, such as the serum, is a useful indicator of both early and late stage prostate cancer.

We have also found that a second HOX protein, HOXB6, and a third protein HOXB5, also show similar results. HOXA3 and HOXD9 are also considered to be useful as biomarkers.

We have further identified the homeodomain-containing transcription factor *Engrailed* 2 (EN-2) as being expressed at both the RNA and protein level in the prostate cancer-derived cell lines LNCaP and PC3, and also in primary prostate cancer cells. Furthermore, the EN-2 protein can be secreted into the surrounding medium of the cells.

5   Surprisingly, elevated levels of all these cancer markers could be detected in prostate cancer patients even when the PSA levels were found to be normal. This further illustrates that the markers of the present invention are more reliable in the diagnosis of prostate cancer when compared to other markers, such as PSA.

Reference to any prior art in the specification is not, and should not be taken as, an
10   acknowledgment, or any form of suggestion, that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be ascertained, understood and regarded as relevant by a person skilled in the art.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude
15   other additives, components, integers or steps.

Thus, in a first aspect, the present invention provides the use of at least one homeodomain-containing transcription factor, or a fragment thereof, as a biomarker for prostate cancer. Examples of homeodomain-containing transcription factors include HOX and EN-2 peptides. The invention also relates to a method for diagnosing prostate cancer disease comprising
20   detecting at least one homeodomain-containing transcription factor in a sample from a patient.

It is particularly preferred that the at least one HOX or EN-2 peptide, or a fragment thereof, is detectable in a body fluid.

Preferably the biomarker is HOXC4 alone. It is also preferred that the biomarker is HOXB5 alone or more preferably HOXB6 alone. It is also preferred that the biomarker is EN-2 alone. It
25   is also preferred that the biomarker is HOXA3 alone. It is also preferred that the biomarker is HOXD9 alone. However, combinations are also preferred. Such combinations include HOXC4 and HOXB5; HOXC4 and HOXB6; HOXB5 and HOXB6; HOXC4, HOXB5 and HOXB6; EN-2 and HOXC4; EN-2 and HOXB5; EN-2 and HOXB6; EN-2, HOXC4 and HOXB5; EN-2, HOXC4 and HOXB6; EN-2, HOXB5 and HOXB6; EN-2, HOXC4, HOXB5 and HOXB6. Such
30   combinations may also further comprise one or both of HOXA3 and/or HOXD9. Further combinations include HOXA3 and HOXD9; HOXA3 and HOXC4; HOXA3 and HOXB5; HOXA3 and HOXB6; HOXA3 and EN-2; HOXD9 and HOXC4; HOXD9 and HOXB5;

HOXD9 and HOXB6; HOXD9 and EN-2. A particularly preferred combination of markers comprises one or more of, preferably all of, HOXC4, HOXB5, HOXB6 and EN-2.

Preferably, the body fluid is the serum or urine. Other preferred body fluids are discussed below.

The HOX genes are a family of homeodomain-containing transcription factors that determine
5   the identity of cells and tissues during early development (reviewed by Morgan, 2006). Recent publications, including Miller *et al* 2003, have effectively identified every single protein that is expressed, and retained intracellularly, in a prostate cancer tumour cell. Of course, this means that a total of around 10,000 to 12,000 proteins, including the HOX peptides, were identified.

However, we have surprisingly discovered that, from amongst these thousands of proteins
10   expressed in prostate cancer cells, the HOXC4 protein, for instance, is found extracellularly and is an excellent biomarker for prostate cancer.

This is surprising for a number of reasons. Firstly, HOXC4 is a transcription factor and, therefore, you would not expect to find it in the serum. Secondly, although other biomarkers for prostate cancer are known, finding a reliable biomarker has remained elusive, see for instance
15   the problems described above in relation to PSA. Indeed, HOXC4 is both an early stage and, in particular, a late stage biomarker, as shown in Figure 1.

Thus, this protein has not been identified as a biomarker for prostate cancer before although it is known to be expressed in prostate cancer cells, along with many thousands of other proteins, many of which could not be described as biomarkers for prostate cancer. This also applies to
20   other HOX peptides including HOXB5 and HOXB6.

Like the HOX peptides, the Engrailed (En) proteins are also homeodomain-containing transcription factors that show a very high degree of functional conservation during development (reviewed by Morgan, 2006). The original *En* gene was characterised in Drosophila, where the *En* mutant fails to form a border between the anterior and posterior wing (Garcia-Bellido and
25   Santamaria, 1972). Vertebrate homologues of *En* likewise have regulatory roles during development, which include limb development and both the early specification and subsequent axonal migrations of the nervous system (Morgan 2006).

In addition to transcriptional regulation, EN proteins have a number of other, apparently unrelated properties that are functionally important. The first of these is the ability to be secreted
30   from cells, and to be internalized by others (Cosgaya et al, 1998; Joliot et al., 1998, reviewed by Morgan, 2006). The exact mechanistic basis for this phenomenon is unknown, although it is

clear that it depends upon a conserved nuclear export sequence located in the homeodomain (Maizel et al., 1999; Chatelin et al., 1996; Derossi et al., 1996; Joliot et al., 1997).

In addition to its developmental role, *EN-2* has recently been shown to be a potential oncogene in breast cancer (Martin et al., 2005). Non-tumorogenic murine mammary cell lines forced to

5   express *EN-2* subsequently exhibit a number of malignant characteristics including a shortening of the cell cycle and a loss of cell to cell contact. Further, RNAi silencing of *EN-2* indicates that it is required for the maintenance of the transformed phenotype in a human breast cancer cell line.

We have discovered that EN-2 is overexpressed and secreted from the prostate cancer derived

10 cell line LNCaP and in human tissue samples from prostatic adenocarcinoma. Furthermore, EN-2 is secreted from ductal cells and adenocarcinoma tumours and is present in the urine of prostate cancer patients. This is the first time that EN-2 has been suggested as a marker of prostate cancer.

Preferably, presence or absence of the at least one homeodomain-containing transcription factor,

15 such as a HOX peptide or EN-2 peptide, or fragment thereof, is detected.

Preferably, the at least one HOX peptide is selected from any HOX peptide as listed below, provided that the HOX peptide, or a fragment thereof, is detectable in a body fluid such as serum or urine.

Preferably, the at least one HOX peptide, or a fragment thereof is from the HOXA, the HOXB,

20 the HOXC or the HOXD subfamily. If more than one HOX peptide is used, then these may be from the same family or from a combination of families. Combinations of fragments are also envisaged, either fragments of the same protein or fragments of different proteins. Combinations of different peptides can provide more accurate diagnoses. It is preferred that the HOX peptide is not HOXA4, HOXA1, HOXA7, HOXB3, or HOXB9.

25 It is particularly preferred that the HOX peptide is the HOXC4 peptide, and/or the HOXB6 peptide and/or the HOXB5 peptide, and/or the HOXA3 peptide, and/or the HOXD9 peptide or fragments thereof.

Preferably, the HOXC4 peptide is that provided in Sequence ID NO. 1 (NCBI accession no. P09017, gi:123279), or a fragment thereof. Preferably, the HOXB6 peptide is that provided in

30 Sequence ID NO. 2 (NCBI accession no. P17509, gi:116242515), or a fragment thereof. Preferably, the HOXB5 peptide is that provided in Sequence ID NO. 3 (NCBI accession no. P09067, gi:400000), or a fragment thereof. Preferably, the EN-2 peptide is that provided in

Sequence ID NO. 4 (NCBI accession no. P19622, gi:21903415) or a fragment thereof. Preferably, the HOXA3 peptide is that provided in Sequence ID NO.5 (mRNA NCBI accession no. NM_030661, gi: 84043946), or a fragment thereof. Preferably, the HOXD9 peptide is that provided in Sequence ID NO.6 (mRNA NCBI accession no. NM_014213, gi: 23397673), or a

5   fragment thereof.

In one embodiment, there is provided a method of diagnosing or monitoring the progression of prostate cancer, comprising detecting and/or quantifying at least one homeodomain containing transcription factor or a fragment thereof in a body fluid, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO: 4, and wherein the fragment has

10   at least 80% sequence homology to said SEQ ID NO: 4.

In another embodiment, there is provided a method of diagnosing prostate cancer comprising quantifying or detecting the amount of at least one homeodomain containing transcription factor, or a fragment thereof, detectable in a body fluid and comparing the amount of said at least one peptide in said test sample with the amount present in a normal control biological sample from a

15   normal, cancer-free, subject, where an increase in the level of the at least one homeodomain containing transcription factor, or a fragment thereof, being found in the test sample is indicative that the disease is either progressing or has initiated, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO. 4, and wherein the fragment has at least 80% sequence homology to SEQ ID NO. 4.

20   In another embodiment, there is provided a method of diagnosing or monitoring the progression of prostate cancer, comprising detecting and/or quantifying RNA encoding a homeodomain containing transcription factor or fragment thereof in a body fluid, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO: 4, and wherein the fragment has at least 80% sequence homology to said SEQ ID NO: 4.

25   While reference is made to HOXC4, the HOXC4 peptide or a fragment thereof, it will be understood that these terms can be used interchangeably, unless otherwise apparent. The same follows for other HOX peptides and EN-2.

Furthermore, where reference is made herein to HOXC4, HOXB6, HOXB5, HOXA3 or HOXD9, this also applies to other HOX peptides, or combinations thereof, unless otherwise

30   apparent.

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Similarly, where reference is made herein to a particular sequence, such as a reference sequence or a Sequence ID NO, this applies to all Sequence ID NOs. and any of the HOX sequences provided below, unless otherwise apparent.

Preferably, the fragment comprises at least 80% sequence homology to the reference sequence (e.g. HOXC4, HOXB6, HOXB5, HOXA3, HOXD9 or EN-2), more preferably 85%, more preferably 90%, more preferably 95%, more preferably 97%, more preferably 99% and most preferably 99.9% sequence homology to the reference sequence, or as close thereto as appropriate. Suitable methods for establishing sequence homology include the BLAST programme.

Preferably, the fragment comprises at least 80% sequence identity to the reference sequence (e.g. HOXC4, HOXB6, HOXB5, HOXA3, HOXD9 or EN-2), more preferably 85%, more preferably 90%, more preferably 95%, more preferably 97%, more preferably 99% and most preferably 99.9% sequence identity to the reference sequence, or as close thereto as appropriate. Suitable methods for establishing sequence identity include the BLAST programme.

Two polypeptides are said to be "homologous", as the term is used herein, if the sequence of one of the polypeptides has a high enough degree of identity or similarity to the sequence of the other polypeptide. "Identity" indicates that at any particular position in the aligned sequences, the amino acid residue is identical between the sequences. "Similarity" indicates that, at any particular position in the aligned sequences, the amino acid residue is of a similar type between the sequences. Degrees of identity and similarity can be readily calculated (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing. Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). Percentage identity, as referred to herein, is as determined using BLAST version 2.1.3 using the default parameters specified by the NCBI (the National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) [Blosum 62 matrix; gap open penalty=11 and gap extension penalty=1].

Preferably, the fragment comprises at least four consecutive amino acids from the reference sequence, more preferably at least 5, more preferably at least 6, more preferably at least 7, more preferably at least 8 consecutive amino acids from the reference sequence, such as Sequence ID NO. 1, 2, 3, 4, 5 or 6, although longer fragments at least 10, 15, 20, 25, 30, 50, 75, 100, 150, 200, 225 and up to at least 250 amino acids are also preferred. Fragments also include truncated

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peptides that have x amino acids deleted from the N-terminus and/or C-terminus. In such truncations, x may be 1 or more (*ie.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more), but preferably less than 150.

Without being bound by theory, it is thought that HOXC4 is secreted via a non-classical route, with no signal sequence required. The sequence given in Sequence ID NO. 1 is, therefore, thought to be the same as the protein found in serum. This is corroborated by the observation that the detected band is of a corresponding size (Mw) to the predicted size based on the gene sequence. HOX proteins may also leave cells through other mechanisms e.g. cell lysis.

The term "biomarker" is used throughout the art and means a distinctive biological or biologically-derived indicator of a process, event or condition. In other words, a biomarker is indicative of a certain biological state, such as the presence of cancerous tissue. In some cases, different forms of biomarkers can be indicative of certain disease states but, without being bound by theory, it is thought that merely the presence of elevated levels of a HOXC4 peptide, or fragment thereof, in body fluids such as serum, is indicative of prostate cancer. Although it is not currently envisaged that different glycoforms, for instance, of the HOXC4 peptide, are secreted, these are nevertheless encompassed by the present invention. For instance, different glycoforms, such as altered glycoform structure or sugar content, may yet be determined for HOXC4, but these are encompassed and may even also be indicative of the progress of the prostate cancer. Truncations, mutations, or deletions of, or ligations to, the HOXC4 peptide, or fragment thereof, are also envisaged.

However, the principal discovery underpinning the present invention is that there is a significant increase in the level of HOXC4 detectable in body fluids, such as the serum. It is thought that HOXC4 is secreted, but it is also envisaged that it may be due to HOXC4 protein leaking out of dying cells. Similarly, EN-2 may be secreted or may be detectable in body fluids due to leaking from damaged or dead cells. Such increased levels are indicative of both early stage and late stage prostate cancer. Whilst there is a significant rise between control or normal levels and early stage prostate cancer, there is also a very significant increase between early and late stage prostate cancer. Broadly, it is an advantage of the present invention that the substance and also the state of the prostate cancer can be detected. This aids in the prognosis and provision of suitable therapies.

It is also an advantage of the present invention that it is able to distinguish over conditions such as benign prostatic hyperplasia BTH (BPH). Such conditions, which lead to an enlargement of the prostatic gland, can be misinterpreted as prostate cancer using established techniques for determining the presence of prostate cancer, such as Digital Rectal Examination (DRE). It is

another advantage of the present invention that an accurate diagnosis can be provided without resorting to unpleasant and potentially harmful invasive procedures, which may also be inaccurate. Furthermore, the present invention is particularly sensitive. Preferably the methods of the present invention may detect the onset of prostate cancer prior to any other detection method and prior to the onset of the overt symptoms of prostate cancer. Thus, the cancer may be treated at an early stage when it is more susceptible to such treatment and less likely to have entered the metastatic stage.

Different types of prostate cancer are known. The most common starts in the prostate gland cells and is known as prostate adenocarcinoma. However, other forms of prostate cancer exist, such as, sarcomas, small cell carcinomas, and transitional cell carcinomas. The methods of the invention may be used to detect the onset of any of these types of cancer, although the detection of adenocarcinoma is preferred.

The progression of cancer is monitored by a staging process. This indicates how well developed the cancer is and if it has spread. The score runs from one to four, with the prognosis becoming progressively worse at each stage.

Stage One: Malignant cells are confined to the prostate; they have not spread to the lymph nodes or other organs; Gleason scores are between two to four, and less than five percent of the prostate is composed of tumor growth.

Stage Two: Gleason scores are five or higher, or over five percent of the gland shows abnormal growth; the cancer is still restricted to the prostate.

Stage Three: Malignant cells have spread to the seminal vesicles, but not to the lymph nodes or other organs.

Stage Four: The lymph nodes, pelvic tissue or more distant organs are affected.

Preferably, the methods of the invention detect the onset of prostate cancer prior to, or during stage one or stage two, more preferably stage one.

The peptide biomarkers of the present invention can be used in methods of diagnosis, for instance clinical screening, and in methods of prognosis assessment, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, drug screening and development. Furthermore, the biomarkers of the present invention and uses thereof are valuable for identification of new drug treatments and for discovery of new targets for drug treatment.

Thus, in a further aspect, the invention provides a method of diagnosing or monitoring the progression of prostate cancer, comprising detecting and/or quantifying at least one

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homeodomain-containing transcription factor such as a HOX peptide or EN-2 peptide or a fragment thereof. Preferably, the peptide comprises or consists of the amino acid sequence according to SEQ ID NO 1, 2, 3, 4, 5 or 6. Preferably the at least one HOX peptide, the EN-2 peptide, or a fragment thereof, is present in a biological sample from a test subject. Said biological sample preferably being a body fluid sample.

The body fluid may be any human body fluid, including cellular fluid, cerebrospinal fluid (CSF), semen, urine, blood, lymph or saliva. However, it is particularly preferred that the body fluid is whole blood, in particular blood serum, or urine. It is also preferred that the body fluid is substantially or completely free of whole/intact cells. Preferably the body fluid is free of platelets and cell debris (such as that produced upon the lysis of cells). Preferably the body fluid is free of both prokaryotic and eukaryotic cells.

Such fluids can be obtained by any number of means known in the art, such as will be apparent to the skilled person. For instance, a urine sample is easily attainable, whilst blood or serum samples can be obtained parenterally by using a needle and syringe, for instance. Cell free or substantially cell free samples can be obtained by subjecting the body fluid to various techniques known to those of skill in the art which include, but are not limited to, centrifugation and filtration.

Although it is generally preferred that no invasive techniques are used to obtain the sample, it still may be preferable to obtain samples such as tissue homogenates, tissue sections and biopsy specimens.

Monitoring methods of the present invention can be used to detect the onset, progression, stabilisation, amelioration and/or remission of prostate cancer. Thus, the detection of the at least one HOX peptide, EN-2 peptide or a fragment thereof, can be used to determine the progression of the disease, in order to direct a physician towards a particular treatment.

Preferably, at least two detection and/or quantification steps are provided, preferably spaced apart temporally, for instance by a few days, weeks, years or, more preferably, months, to determine whether the levels of the at least one HOX or EN-2 peptide, or a fragment thereof, have changed, thus indicating whether there has been a change in the progression of the cancer. This enables comparisons to be made between a level of the biomarker in samples taken on two or more occasions, as an increase in the level of the peptide over time is indicative of the onset or progression of the cancer, whereas a decrease in the level of the peptide may indicate amelioration and/or remission of the cancer.

Preferably, a method of diagnosis or of monitoring according to the present invention comprises comparing the level of the peptide present in a test sample, with the level in one or more

controls. Preferably, the controls may be from the same patient from a previous sample, to thus monitor onset or progression. However, it is also preferred that the controls may be normalised for a population, particularly a healthy or normal population, where there is no prostate cancer. In other words, the control may consist of the level of a biomarker found in a normal control sample from a normal subject.

Thus, there is provided a method of diagnosing prostate cancer comprising quantifying or detecting the amount of at least one HOX peptide, the EN-2 peptide or a fragment thereof, and comparing the amount of said peptide in said test sample with the amount present in a normal control biological sample from a normal subject or from the same subject well prior to the incidence of disease. If a significant increase in the level of the at least one HOX peptide, the EN-2 peptide or a fragment thereof, is found in the test sample, then it is indicative that the disease is either progressing or has initiated.

As noted above, this method of detection of at least one HOX peptide, the EN-2 peptide or a fragment thereof is particularly useful in detecting early stage prostate cancer and is more sensitive than the traditional test of detecting PSA levels. Thus, the methods of the invention are particularly useful for confirming cancer when a patient has tested negative for cancer using conventional methods, such as the detection of PSA.

In general, the level of the at least one HOX peptide or EN-2 peptide secretion into the body fluids is in the ranges described below.

It will be understood that the terms "peptide" and "protein" are interchangeable as used herein, unless otherwise apparent.

Table 1 and Figure 1, for instance, show a very sizeable increase in HOXC4 serum protein concentrations. Table 2 and Figure 2 show similar results for HOXB6. We have also shown that HOXB6 is a biomarker for late-stage prostate cancer (data not shown). HOXB5 is also a biomarker, but is less preferred than HOXB6 or HOXC4 (Table 3 and Figure 7). Other alternative biomarkers include HOXA3 and HOXD9 (see Figure 8).

Preferably, the increase is statistically significant, determined by using standard methods, such as a "t-test" providing confidence intervals of preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97%, more preferably at least 99%, more preferably at least 99.5%, more preferably at least 99.95%, and most preferably at least 99.99%.

Preferably, the increase between the control and a sample indicative of early stage prostate cancer is around between 110-200%, more preferably around between 120-180%, more

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preferably around between 130-160%, more preferably around between 140-150%, more preferably around between 110-140%, more preferably around between 115-130%, more preferably around between 120-130%, more preferably around between 120-140%, and most preferably around 125%. Other combinations of the above end points are also envisaged.

With regard to determining late stage or advanced cancer, the increase between the control and a sample indicative of said late stage prostate cancer is around between 400-800%, more preferably around between 450-750%, more preferably around between 500-700%, more preferably around between 520-680%, more preferably around between 540-640%, more preferably around between 550-620%, more preferably around between 560-600%, more preferably around between 570-590%, and most preferably around 580%. The increase may also be in the range 550-800% or 400-600%. Other combinations of the above end points are also envisaged.

An increase between early and late stage cancer may be measured relative to a normal (i.e. cancer free) control or to an early stage sample or control in the same patient. Relative to an early stage sample an increase of around between 100% and 300%, more preferably around between 150% and 250%, more preferably around between 175% and 225%, more preferably around between 185% and 215%, more preferably around between 100% and 220%, more preferably around between 170% and 300%, and most preferably around 200% is preferred.

As mentioned above, where reference is made herein to at least one homeodomain –containing peptide, this applies to HOX peptides, such as HOXC4, HOXB6, HOXB5, HOXA3 and HOXD9, and to the EN-2 peptide, unless otherwise apparent.

Where reference is made herein to at least one HOX peptide or HOX peptides, this applies to HOXC4, HOXB6, HOXB5, HOXA3 and HOXD9, in particular as well as the other HOX peptides listed below, unless otherwise apparent.

In terms of absolute amounts of at least one HOX or EN-2 protein per ml of body fluid, preferably serum, this is preferably around between 0.5-1.5 ng/ml, more preferably around between around between 0.75-1.25 ng/ml, and most preferably around 1.0 ng/ml for control samples. For early stage cancer samples, preferred ranges are around between 2.0-5.0 ng/ml, more preferably around between 2.5-4.5 ng/ml, more preferably around between 2.5-4.0 ng/ml, more preferably around between 2.75-3.5 ng/ml, more preferably around between 2.75-3.0 ng/ml, more preferably around between 2.0-3.5 ng/ml, more preferably around between 2.0-3.0 ng/ml, and most preferably around 3.0 ng/ml.

For late stage cancer samples, preferred ranges are around between 6.0-20 ng/ml, more preferably around between 8.0-15 ng/ml, more preferably around between 8.5-13 ng/ml, more

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preferably around between 9.0-12 ng/ml, more preferably around between 9.5-11 ng/ml, more preferably around between 7-13 ng/ml, more preferably around between 8.5-16 ng/ml, and most preferably around 10 ng/ml.

The term "diagnosis" encompasses identification, confirmation, and or characterisation of the presence of absence or absence of prostate cancer, together with the developmental stage thereof, such as early stage or late stage, or benign or metastatic prostate cancer.

It will be appreciated that the "early stage" and "late stage" nature of the cancer disease states can be determined by a physician. It is also envisaged that they can refer to non-metastatic and metastatic, respectively.

The invention also provides a method of monitoring the efficacy of a treatment for prostate cancer, comprising detecting and/or quantifying the at least one HOX peptide, EN-2 peptide, or a fragment thereof, present in a biological sample from a subject. As above, it may be preferable to obtain two samples, separated temporally by, for instance, a couple of months, in order to determine whether there has been any progress in the treatment, in other words, if the levels of at least one HOX peptide, EN-2 peptide, or a fragment thereof, have increased or decreased. A decrease in the levels of said at least one HOX peptide or EN-2 peptide indicates that the disease is regressing and thus the treatment regime is successful. Such a monitoring of a disease will quickly show whether a patient is responding to a treatment or is refractive to a treatment. If the latter, then an alternative treatment may be provided. Such a method therefore provides a fast indication of whether a treatment regime is effective and allows the physician to alter such a regime until an effect is observed in the patient.

In the method of the present invention, it is preferred that the amount of the peptide, or a fragment thereof, in a test sample is compared with the amount present in one or more control(s) and/or one or more previous test sample(s) taken at an earlier time from said same test subject. The length of time between the earlier and later samples may be only a few days, but may be up to several months or even several years, and will be determined by the physician, based on the progression of the disease and the patient's medical history. Alternatively, the control may be a test sample from a disease-free patient.

The peptide, or a fragment thereof, is detected by confirming the presence of said biomarker. Quantifying the amount of the biomarker present in a sample preferably includes determining the concentration of the peptide biomarker in the sample. The detecting and/or quantifying steps can be performed directly on the sample, or indirectly on an extract therefrom, or a dilution thereof.

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The present invention relates to biomarkers and it will be appreciated that these have to be detectable in the body fluid. Some members of the HOX family may not be detectable in body fluid and are, therefore not biomarkers according to the present invention.

Detectable levels of the at least one HOX peptide, the EN-2 peptide or fragments thereof are discussed elsewhere, but it will be appreciated that there may be a number of reasons why a HOX peptide, the EN-2 peptide or fragments thereof are not detectable. Without being bound by theory, one reason may be that the peptide is degraded in the body fluid, i.e. is not "stable."

Thus, it is preferred that the at least one HOX peptide, the EN-2 peptide or fragments thereof are substantially not degraded and/or are still detectable (i.e. present at detectable concentrations) at the body periphery, i.e. a vein near the surface of the skin, preferably the arm, where it can be more simply sampled.

The peptide may be released into the body fluid, either by cell break-up or leakage, or by secretion.

Detection and/or quantification of the at least one HOX peptide or the EN-2 peptide, or fragments thereof may be by any method suitable to detect the presence and/or amount of a specific protein or peptide in a biological sample. This may be by direct detection of the biomarker, by instance by MALDI-TOF or SELDI.

However, it is also preferred that the at least one HOX peptide or EN-2 peptide, or fragment thereof, is detected directly or indirectly via interaction with a ligand or ligands. Such ligands may include an anti-HOX or anti-EN-2 antibody or a binding fragment thereof, an aptamer, or an oligonucleotide. It will be appreciated that these ligands must be capable of specifically binding the HOX peptide or EN-2 peptide, or a fragment thereof. It may be suitably labelled and comprise an affinity tag. Suitable labels may be fluorescent, radioactive or luminescent. The labels may be attached to the antibody directly, or via a linker.

For example, detecting and/or quantifying may be performed by one or more methods selected from the group consisting of: MALDI-TOF, SELDI, 1-D or 2-D gel-based analysis systems, Liquid Chromatography combine liquid chromatography and Mass spectrometry techniques. The latter of these may include, preferably, ICAT®, or iTRAQ® (both available from Applied Biosystems, USA).

Liquid chromatographic techniques may include HPLC (high pressure liquid chromatography) or even low pressure liquid chromatography (LPLC). Furthermore, methods such as thin-layer chromatography, NMR spectroscopy and any other method described herein, are also preferred.

The peptide, or a fragment thereof, may be detected and/or quantified using immunological methods, such as sandwich immunoassays, for instance an enzyme linked immunosorbent assay (ELISA), radio-immunoassays (RAI), enzyme immunoassays (EIA), Western Blotting, immunoprecipitation. Also preferred are particle-based immunoassays, which may include, for instance, using gold, silver, or latex particles, magnetic particles or Q-dots. Such methods may be performed, for example, in microtitre plate or strip format or on a "chip".

Particularly preferred are ELISAs comprising antibodies specific for the at least one HOX or EN-2 peptide. The anti-HOX or anti-EN-2 antibody or antibodies are preferably linked, either directly or via a linker, to a reporter molecule, such as a radioisotope, a fluorescent molecule, a luminescent molecule or an enzyme. One example of a preferred enzyme is alkaline phosphatase, available from Invitrogen, UK, although other reporters are known in the art.

An example of anti-human HOXC4 antibody is available from Abcam, UK, Catalogue No. ab24338. This is rabbit anti-human HOXC4 antibody and, therefore, it may be preferable to use a secondary, anti-rabbit IgG antibody linked to a reporter, such as alkaline phosphatase, to detect the HOXC4 antibody-complex. An example of an anti-HOXB6 antibody is from Abcam, cat no. ab26077. An example of an anti-HOXB5 antibody is from Abcam, cat no. ab26079. An example of an anti-HOXA3 antibody is from Abcam, cat no. ab28771. An example of an anti-HOXD9 antibody is from Abcam, cat no. ab60708. Examples of other anti-HOX antibodies are known. An example of an anti-EN-2 antibody is from Abcam, cat no. ab45867.

Western blotting methods are also preferred, particularly as these can provide densitometry data.

Suitable comparative levels of the presence of the at least one HOX or EN-2 peptide sufficient to distinguish between the control, early-stage and late-stage cancers, or combinations thereof, are discussed above.

Where antibodies are used, these may be polyclonal, monoclonal, bispecific, humanised or chimeric antibodies. The antibodies may consist of a single chain but would preferably consist of at least a light chain or a heavy chain but it will be appreciated that at least one Complementarity Determining Region (CDR) is required in order to bind a HOX epitope or an EN-2 epotope.

Of course, it will also be appreciated that the epitope or epitopes must be available for binding or recognition by the ligand, such as an antibody. Anti-HOX and anti-EN-2 antibodies are already known, but if further antibodies or binding ligands, with different specificities are required then these could be obtained. Methods of making antibodies are known in the art. For example, if polyclonal antibodies are desired, then a selected mammal, such as a mouse, rabbit, goat or horse may be immunised with the antigen of choice, such as human HOX or EN-2. The

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serum from the immunised animal is then collected and treated to obtain the antibody, for instance by immunoaffinity chromatography. Human HOX or EN-2 could, for instance, be injected into rabbits triggering a rabbit immune response, raising anti-HOX or anti-EN-2 antibodies, which can thereby be isolated, using standard methods.

Of course, monoclonal antibodies could be produced by methods known in the art, and are generally preferred. The general methodology for making monoclonal antibodies using hybridoma technology is well known (see, for example, Kohler, G. and Milstein, C., Nature 256: 495-497 (1975); Kozbor et al., Immunology Today 4: 72 (1983); Cole et al., 77-96 in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. (1985).

Therefore, an antibody, as referred to herein, must consist of an epitope-binding region, such as CDR. As used herein, the term "antibody" refers to intact molecules as well as to fragments thereof, such as Fab, Fab', F(ab')$_2$ and Fv, which are capable to binding to an epitope. Preferably, the antibody also comprises an effector portion, such as the Fc region.

The antibody may of any suitable class, including IgE, IgM, IgD, IgA and, in particular, IgG. The various subclasses of these antibodies are also envisaged.

Thus, it is also preferred that a biosensor is used to detect the at least one peptide biomarker, or a fragment thereof. The biosensor may incorporate an immunological method, as described above, for instance, for detection of the biomarker, or electrical, thermal, magnetic, optical for instance a hologram, or acoustic means or technologies. Using such biosensors, it is envisaged that the biomarker is detected and/or quantified. Thus, in a further aspect, the present invention provides the use of a biosensor to detect and/or quantify the peptide, or a fragment thereof.

Preferably, the at least one HOX or EN-2 biomarker can be detected using a biosensor based on the use of "smart" holograms, or high frequency acoustic systems, particularly as such systems are amenable to array configurations. For instance, with smart hologram sensors, available from Smart Holograms Limited, Cambridge UK, a holographic image is stored in a thin polymer film that is sensitised to react specifically with the biomarker. On exposure, the biomarker reacts with the polymer leading to an alteration in the image displayed by the hologram. The test result readout can see a change in the optical brightness, image, colour and/or position of the image. This can even be read by eye.

It will be appreciated that it is preferred that the biosensors capable of detecting and/or quantifying the at least one HOX or EN-2 biomarker, combine biomolecular recognition with appropriate means to convert detection of the presence, or quantification, of said biomarker in a sample into a detectible signal. For instance, imprinted recognition elements, thin film transistor technology, magnetic acoustic resonator devices and other novel acousto-electrical

systems may preferably be employed in the present biosensor for detection and/or quantification of the at least one HOX or EN-2 biomarker. Preferably, a biosensor according to the invention will detect one or more of, preferably two, three, four, five or all six of, HOXC4, HOXB5, HOXB6, HOXA3, HOXD9 and EN-2. Further preferred biosensors may also further detect other HOX biomarkers as referred to above.

Preferably, the biosensor includes a ligand and/or an antibody, as discussed above. The biosensor may be provided on a suitable array or "chip", such that the biosensor is capable of specific binding to the at least one HOX peptide, or EN-2 peptide, or a fragment thereof, a detection means is provided. Suitable detection means are discussed above, but may include reporter systems, such as alkaline phosphatase, or smart holograms, capable of providing a detectible signal to the user to indicate that specific binding of the at least one HOX or EN-2 peptide, or a fragment thereof, has been achieved and, preferably, an indication of the concentration of HOX or EN-2 or the number of specific binding events that have occurred on said array or chip.

The present invention further provides kits for said detection and/or quantification. Such kits are useful in the diagnosis and/or monitoring of prostate cancer. Thus, such kits will preferably comprise a biosensor such as a ligand, preferably an antibody, capable of specifically binding at least one peptide, or a fragment thereof, and means for indicating said specific binding for the user, for instance using a reporter system as described herein. Particularly referred kits include an array or a chip.

In a further aspect, the invention provides the use of a biosensor, preferably a ligand and most preferably an antibody, capable of specific binding and recognition of at least one HOX or EN-2 peptide, or a fragment thereof, to identify a substance capable of suppressing the generation of the at least one HOX or EN-2 peptide, or a fragment thereof. Preferably, this substance is capable of suppressing the production of the biomarker by the prostate cancer. In other words, the ligand may be used to determine the efficacy or otherwise of a putative or established anti-prostate cancer treatment.

Thus, a method for detecting the efficacy of a putative anti-cancer treatment may include the steps of:

(a) incubating a whole cell expressing a HOX peptide or an EN-2 peptide and detecting the amount of HOX peptide or an EN-2 peptide released,

(b) treating the cell with a putative anti-cancer treatment,

(c) detecting the amount of HOX peptide or an EN-2 peptide released following treatment, and

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(d) comparing the amount of HOX peptide or an EN-2 peptide released both before and after treatment to see whether the putative anti-cancer treatment has an effect on the amount of peptide released.

Alternatively, a method may comprise the steps of:

(a) incubating a labelled ligand with a whole cell expressing a HOX peptide or an EN-2 peptide on the cell surface, or a cell membrane containing a HOX peptide or an EN-2 peptide,

(b) measuring the amount of labelled ligand bound to the whole cell or the cell membrane;

(c) adding a putative anti-cancer agent to a mixture of labelled ligand and the whole cell or the cell membrane of step (a) and allowing the mixture to attain equilibrium;

(d) measuring the amount of labelled ligand bound to the whole cell or the cell membrane after step (c); and

(e) comparing the difference in the labelled ligand bound in step (b) and (d), such that the compound which causes the reduction in binding in step (d) is considered to be an inhibitor of HOX or EN-2 expression and therefore an anti-cancer agent.

For example, a cell line such as the prostate carcinoma cell line LNCaP could be used in such an assay. The LNCaP cells could be grown in the presence or absence of a putative or established anti-cancer agent, and the relative expression of EN-2 compared. A lower expression level of EN-2 would indicate a better anti-cancer activity. The expression levels could be detected by detecting the protein produced (*i.e.* the final product) or by detecting the RNA produced (*i.e.* an intermediate product). Methods of detecting the protein product are discussed above. The RNA may be detected using, for example, quantitative PCR or an oligonucleotide array.

The above also relates to the methods and kits of the present invention.

Other methods or kits for use in accordance with the present invention, will be apparent to the skilled person. These may include, for instance, biomarker detection systems are disclosed in US-2006-014301, US-2004-063216, WO 01/92879 and WO 02/054936.

Thus, the present invention can be used to identify new anti-prostate cancer treatments, wherein the levels of at least one HOX or EN-2 peptide, or a fragment thereof, are detected and/or quantified in the presence or absence of the putative anti-cancer agent, in order to determine the efficacy thereof. The present invention can also be used in a method of high-throughput screening technologies, for instance in a configured array format, such as on a chip or in a multiwell array.

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As prostate cancer is a male-related disease, it will be appreciated that the subject or patient is a male.

Description of the Figures

Figure 1: An example of a western blot looking for HOXC4 protein in serum. The results were also analysed by densitometry, and are shown as the mean of all six samples from each group. Error bars show the standard error of the mean. These data indicate that HOXC4 is potentially a specific and accurate diagnostic marker for prostate cancer at both early and late stages of the disease.

Figure 2: Detection of HOXB6 in six patients with early stage prostate cancer where tumours are small and confined to the prostate, and from six age-matched controls.

Figure 3: (a) RT-QPCR analysis of EN-2 expression in LNCaP and primary tumour cells (transcript level as a proportion of β-actin). (b,c) Core biopsy of a prostatic adenocarcinoma stained with anti-En2 antibody. En2 positive staining (brown) is present in the cytoplasm of tumour cells, with strongest staining at the luminal border (1). En2 positive blebs are visible attached to the luminal border (2) or free within the lumen (3). The nuclei are stained blue. Magnification: (a) x100, (b) x60.

Figure 4: Sections through TMA core biopsies of (a) normal prostate tissue, (b) benign hypertrophy of the prostate, and (c and d) malignant prostate tumour. EN-2 staining (brown) is present in the stromal cells (white filled arrow) and in the glands with strongest staining apparent at the luminal border (filled arrow heads). The nuclei are stained blue. Magnification: (a-c) x60, (d) x100.

Figure 5: Western blot detection of EN-2 protein in culture medium.

Figure 6: EN-2 protein in urine. Urine was collected from individuals being examined for symptoms consistent with prostate cancer and screened for the presence of EN-2 protein. The presence of absence of cancer was tested for by biopsy. The predicted MW of EN-2 is 30kDa but a second band of 33kDa is also detected in the positive control (mouse cerebellum extract). The 30kDa band is exclusively present in the urine of 50% of patients that had prostate cancer cells in their biopsy (C Pa), it was not present in patients with negative biopsies or with benign hypertrophy of the prostate (BHP) or high grade prostatic intraepithelial neoplasia (HGPIN).

Figure 7: Detection of HOXB5 in three patients with early stage prostate cancer where tumours are small and confined to the prostate, three patients with late stage prostate cancer and from three age-matched controls.

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Figure 8: Expression levels of various HOXA and HOXD genes in tumour tissue compared to normal adjacent tissue. DU145 and PC3 are cell lines derived from prostate tumours.

Example 1

HOXC4

Serum was obtained from six patients with advanced, hormone-refractory prostate cancer, six patients with early stage prostate cancer where tumours are small and confined to the prostate, and from six age-matched controls. This was de-salted and fractionated using a 12.5% PAGE/SDS gel. Human HOXC4 protein (UniProtKB / Swiss-Prot entry number P09017) was detected using western blotting with a rabbit anti-human HOXC4 antibody (Abcam, UK, catalogue number ab24338), together with a secondary anti-rabbit IgG antibody linked to alkaline phosphatase (Invitrogen, UK). Using this method, we detected high levels of HOXC4 protein in the serum of all the prostate cancer patients, but only very low levels in age-matched control serum (Fig 1).

The results are shown below in Table 1.

HOXC4 could be used as the basis of a diagnostic and / or prognostic test for prostate cancer by using a standard assay technique such as ELISA. This would involve the use of an anti-HOXC4 antibody linked to a reporter such as alkaline phosphatase in order to quantify the amount of HOXC4 protein in patient serum samples. Other methodologies could also be used, including western blotting. The amount of HOXC4 protein would provide an indication as to whether the disease is present and what stage it has reached.

Gel name : HoxC4 blot 131006 (Raw 1-D Image)						
Index	Name	Patient	Diagnosis	Volume OD*mm2	Adj. Vol. OD*mm2	% Adj. Vol.
1	U1	HR29	Cancer	6.844796	0.272431	24.320686
2	U2	HR28	Cancer	6.460027	0.092836	8.2877258
3	U3	HR27	Cancer	9.731784	0.393355	35.115964
4	U4	SR5	Early cancer	6.108735	0.086538	7.7255409
5	U5	SR2	Early cancer	6.26387	0.01121	1.0007083
6	U6	SR1	Early cancer	8.427193	0.15222	13.589161
7	U7	VR30	Healthy	6.459172	0.045458	4.0581642
8	U8	VR23	Healthy	9.760506	0.062926	5.6175665
9	U9	VR21	Healthy	6.740683	0.003187	0.2844833

NB. Probed membranes with primary antibody for 1 hour only

Mean values				% increase
		sd	sem	
Control	0.037190112	0.030716	0.0177548	0
Early cancer .	0.08332277	0.07056	0.0407864	124.0455
Late cancer	0.252873795	0.151211	0.0874053	579.949

Table 1

SEQ ID NO. 1:

HOXC4 NCBI Accession # P09017

```
1    MIMSSYLMDS NYIDPKFPPC EEYSQNSYIP EHSPEYYGRT RESGFQHHHQ ELYPPPPPRP

61   SYPERQYSCT SLQGPGNSRG HGPAQAGHHH PEKSQSLCEP APLSGASASP SPAPPACSQP

121  APDHPSSAAS KQPIVYPWMK KIHVSTVNPN YNGGEPKRSR AAYTRQQVLE LEKEFHYNRY

181  LTRRRRIEIA HSLCLSERQI KIWFQNRRMK WKKDHRLPNT KVRSAPPAGA APSTLSAATP

241  GTSEDHSQSA TPPEQQRAED ITRL
```

Example 2

HOXB6

The detection of HOXB6 in these samples was done exactly as for HOXC4 except that the primary antibody was rabbit-anti human HOXB6 (from Abcam, cat no. ab26077). The relative values for HOXB6 protein were assessed by densitometry of the X-ray film (using the bands located at its predicted molecular weight). These values are the optical density of the band relative to that of the local background. Therefore, it will be appreciated that a negative comparative densitometry value is possible, as shown for the Age Matched Controls (AMCs), although it will be appreciated that this does not translate to a negative amount of HOXB6 protein.

These data are shown graphically in Figure 2.

Table 2

HOXB6 in serum					
Values are relative optical density of bands					
			mean	sd	sem
AMC1	-0.02671	AMC	-0.09016	0.064043	0.036977
AMC2	-0.08899	Early cancer	0.261033	0.076253	0.044026
AMC3	-0.15478				
Early1	0.182775				
Early2	0.33511				
Early3	0.265214				

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SEQ ID NO. 2: HOXB6 NCBI Accession # P17509

```
1    MSSYFVNSTF PVTLASGQES FLGQLPLYSS GYADPLRHYP APYGPGPGQD KGFATSSYYP

61   PAGGGYGRAA PCDYGPAPAF YREKESACAL SGADEQPPFH PEPRKSDCAQ DKSVFGETEE

121  QKCSTPVYPW MQRMNSCNSS SFGPSGRRGR QTYTRYQTLE LEKEFHYNRY LTRRRRIEIA

181  HALCLTERQI KIWFQNRRMK WKKESKLLSA SQLSAEEEEE KQAE
```

Example 3

EN-2

Using quantitative PCR (QPCR) the expression levels of EN-2 were tested in the human prostate carcinoma cell line LNCaP (Horoszewicz et al., 1983). This is a cell line derived from a metastatic lesion of a human prostate adenocarcinoma, and is widely used as an *in vitro* model of this cancer. Furthermore the expression of EN-2 was tested in tissue obtained from biopsies of prostate tumours. Both samples expressed EN-2. The primary tumour cells also expressed *EN-2* as protein. This could be detected in sections of prostatic adenocarcinomas (Fig 3a). EN-2 staining in these sections is present in both the cytoplasmic and nuclear compartments but is most intense at the luminal borders and in secretory blebs entering the lumen (Fig 3b,c).

To further examine the extent to which EN-2 is present in tissue from normal prostate, benign hypertrophy and malignant tumours, we stained a tissue microarray (TMA) containing representative sections from each with an anti-EN-2 antibody. This confirmed that EN-2 is expressed strongly in malignant glands, especially in the luminal borders (Fig 4), although there is some staining in glands from normal and hypertrophic tissue. All tissues also showed EN-2 staining in the stromal cells (Fig 4). In order to quantify staining in the glands each tissue was scored from 0 to 3 where 0 represents no staining and 3 the strongest staining. On this basis glands from normal tissue (Fig 4a) scored a mean value of 0.5 (n=10, sd=0.52), from benign hyperplasia (Fig 4b) 0.67 (n=6, sd=0.51), and from malignant tumours (Fig 4c&d) 2.3 (n=6, sd=0.52).

These data clearly show that EN-2 is expressed in these prostate cancer cells.

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Example 4

The medium surrounding LNCaP and primary prostate tumour cells was examined to establish whether EN-2 can be secreted from these cells. Medium, used to culture both cell types for two hours, was desalted and concentrated. Proteins contained within this concentrate were then resolved on an acrylamide gel. Western blotting revealed the presence of EN-2-specific bands in the medium surrounding both types of cells (Fig 5), and quantification using standard amounts of *in vitro*-transcribed EN-2 protein indicated that the amount of EN-2 in the medium used to culture LNCaP cells was 3ng/ml, whilst primary tumour cells produced 1.75ng/ml in 48 hours.

Example 5

Urine samples were obtained from 17 individuals, who had recently undergone prostatic biopsy and massage. Of these individuals, 10 were found to have cancer upon biopsy. Of the others, two had high grade prostatic intraepithelial neoplasia (HGPIN), three showed signs of benign hypertrophy and two had no cancerous cells in the biopsy section. Five of the patients diagnosed with cancer had large amounts of EN-2 in their urine, while none of the urine samples of the individuals not diagnosed with cancer contained a significant amount of EN-2 protein (Fig 6).

Example 6

HOXB5

The detection of HOXB5 in these samples was done exactly as for HOXC4 and HOXB6 except that the primary antibody was rabbit-anti human HOXB5 (from Abcam, cat no. ab26079). The relative values for HOXB5 protein were assessed by densitometry of the X-ray film (using the bands located at its predicted molecular weight). These values are the optical density of the band relative to that of the local background.

Table 3

Mean values			sd	sem	%increase
	AMC	0.006597	0.005948	0.003438	0
	Early ca	0.244012	0.055004	0.031794	3598.808
	Late ca	0.471597	0.154221	0.089145	7048.621

These data are represented graphically in Figure 7.

<u>Example 7</u>

Urine samples were obtained from 17 individuals, who were suspected to have prostate cancer and who were subsequently diagnosed with the disease. The protein levels of EN2 in the urine samples was assessed as described above. The urine samples of 8 (47%) of these individuals showed elevated levels of EN2. In contrast, in urine samples obtained from 15 patients who were suspected to have prostate cancer, but where the cancer was not confirmed subsequently, none (0%) had elevated levels of EN2. It is noteworthy that 3 of the patients diagnosed with prostate cancer, who showed elevated levels of EN2 in their urine samples, did not have elevated levels of PSA. This illustrates that the methods of the invention are effective at detecting cancer where conventional detection methods have failed.

<u>Example 8</u>

Detection of expression of HOX genes in normal and tumour tissue.

RNA Extraction: Tumour and normal adjacent tissue RNA was purchased from Ambion, USA. RNA from cell cultures was extracted using the RNeasy Mini Kit (Qiagen, UK).

cDNA Synthesis: RNA was first denatured by heating to 65°C for five minutes. 1-5mg of RNA was incubated in a volume of 50ml at 37°C for one hour with final concentrations of 10mM DTT, 1mM dNTPmix, as well as 100mg/ml polyT primers, 200 units of reverse transcriptase (Invitrogen, USA) and 40 units of RNASEout (Invitrogen, USA). The cDNA synthesis reaction was terminated by placing tubes at 65°C for five minutes.

Real-Time PCR: Semi-quantitative RT-PCR was performed using the Stratagene MX4000 Real Time PCR machine. The Stratagene MX4000 measures PCR product accumulation during the exponential phase of the reaction, prior to the amplification becoming vulnerable to limited reagents and cycling variability. Fluorescence increases in accordance with increasing levels of PCR product. The relative amount of each transcript was determined as a ratio with the amount of Beta-actin transcript in each sample.

The primers used are listed in table 4 below.

Gene	Forward primer	Reverse primer
HOXA1	CTGGCCCTGGCTACGTATAA (SEQ ID NO:7)	TCCAACTTTCCCTGTTTTGG (SEQ ID NO:8)
HOXA2	TTCAGCAAAATGCCCTCTCT (SEQ ID NO:9)	TAGGCCAGCTCCACAGTTCT (SEQ ID NO:10)
HOXA3	ACCTGTGATAGTGGGCTTGG (SEQ ID NO:11)	ATACAGCCATTCCAGCAACC (SEQ ID NO:12)
HOXA4	CCCTGGATGAAGAAGATCCA (SEQ ID NO:13)	AATTGGAGGATCGCATCTTG (SEQ ID NO:14)
HOXA5	CCGGAGAATGAAGTGGAAAA (SEQ ID NO:15)	ACGAGAACAGGGCTTCTTCA (SEQ ID NO:16)

HOXA6	AAAGCACTCCATGACGAAGG (SEQ ID NO:17)	TCCTTCTCCAGCTCCAGTGT (SEQ ID NO:18)
HOXA7	TGGTGTAAATCTGGGGGTGT (SEQ ID NO:19)	TCTGATAAAGGGGGCTGTTG (SEQ ID NO:20)
HOXA9	AATAACCCAGCAGCCAACTG (SEQ ID NO:21)	ATTTTCATCCTGCGGTTCTG (SEQ ID NO:22)
HOXA10	ACACTGGAGCTGGAGAAGGA (SEQ ID NO:23)	GATCCGGTTTTCTCGATTCA (SEQ ID NO:24)
HOXA11	CGCTGCCCCTATACCAAGTA (SEQ ID NO:25)	GTCAAGGGCAAAATCTGCAT (SEQ ID NO:26)
HOXA13	GGATATCAGCCACGACGAAT (SEQ ID NO:27)	ATTATCTGGGCAAAGCAACG (SEQ ID NO:28)
HOXD1	TTCAGCACCAAGCAACTGAC (SEQ ID NO:29)	TAGTGGGGGTTGTTCCAGAG (SEQ ID NO:30)
HOXD3	CAGCCTCCTGGTCTGAACTC (SEQ ID NO:31)	ATCCAGGGGAAGATCTGCTT (SEQ ID NO:32)
HOXD4	TCAAATGTGCCATAGCAAGC (SEQ ID NO:33)	TCCATAGGGCCCTCCTACTT (SEQ ID NO:34)
HOXD8	TCAAATGTTTCCGTGGATGA (SEQ ID NO:35)	GCTCTTGGGCTTCCTTTTTC (SEQ ID NO:36)
HOXD9	TCCCCCATGTTTCTGAAAAG (SEQ ID NO:37)	GGGCTCCTCTAAGCCTCACT (SEQ ID NO:38)
HOXD10	GCTCCTTCACCACCAACATT (SEQ ID NO:39)	AAATATCCAGGGACGGGAAC (SEQ ID NO:40)
HOXD11	GGGGCTACGCTCCCTACTAC (SEQ ID NO:41)	GCTGCCTCGTAGAACTGGTC (SEQ ID NO:42)
HOXD12	CGCTTCCCCCTATCTCCTAC (SEQ ID NO:43)	CTTCGGGCGCATAGAACTTA (SEQ ID NO:44)
HOXD13	GGGGATGTGGCTCTAAATCA (SEQ ID NO:45)	AACCTGGACCACATCAGGAG (SEQ ID NO:46)

The results are shown in Figure 8.Using this method, HOXA3 and HOXD9 were identified as potential biomarkers for prostate cancer, as these genes are clearly overexpressed in tumour tissue samples.

Sequence ID NO. 5 (HOXA3 polypeptide)

MQKATYYDSSAIYGGYPYQAANGFAYNANQQPYPASAALGADGE
YHRPACSLQSPSSAGGHPKAHELSEACLRTLSAPPSQPPSLGEPPLHPPPPQAAPPAP
QPPQPAPQPPAPTPAAPPPPSSASPPQNASNNPTPANAAKSPLLNSPTVAKQIFPWMK
ESRQNTKQKTSSSSSGESCAGDKSPPGQASSKRARTAYTSAQLVELEKEFHFNRYLCR
PRRVEMANLLNLTERQIKIWFQNRRMKYKKDQKGKGMLTSSGGQSPSRSPVPPGAGGY
LNSMHSLVNSVPYEPQSPPPPFSKPPQGTYGLPPASYPASLPSCAPPPPPQKRYTAAGA
GAGGTPDYDPHAHGLQGNGSYGTPHIQGSPVFVGGSYVEPMSNSGPALFGLTHLPHAA
SGAMDYGGAGPLGSGHHHGPGPGEPHPTYTDLTGHHPSQGRIQEAPKLTHL

Sequence ID NO.6 (HOXD9 polypeptide)

MSSSGTLSNYYVDSLIGHEGDEVFAARFGPPGPGAQGRPAGVAD
GPAATAAEFASCSFAPRSAVFSASWSAVPSQPPAAAAMSGLYHPYVPPPPLAASASEP
GRYVRSWMEPLPGFPGGAGGGGGGGGGGPGRGPSPGPSGPANGRHYGIKPETRAAPAP
ATAASTTSSSSTSLSSSSKRTECSVARESQGSSGPEFSCNSFLQEKAAAATGGTGPGA
GIGAATGTGGSSEPSACSDHPIPGCSLKEEEKQHSQPQQQQLDPNNPAANWIHARSTR
KKRCPYTKYQTLELEKEFLFNMYLTRDRRYEVARILNLTERQVKIWFQNRRMKMKKMS
KEKCPKGD

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of diagnosing or monitoring the progression of prostate cancer, comprising detecting and/or quantifying at least one homeodomain containing transcription factor or a fragment thereof in a body fluid, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO: 4, and wherein the fragment has at least 80% sequence homology to said SEQ ID NO: 4, and wherein the fragment comprises at least ten consecutive amino acids from said SEQ ID NO: 4.

2. A method according to claim 1, wherein the at least one EN-2 peptide or a fragment thereof, is present in a biological sample from a test subject.

3. A method according to any one of the preceding claims, wherein the body fluid is cellular fluid, cerebrospinal fluid (CSF), semen, urine, blood or saliva.

4. A method according to claim 3, wherein the body fluid is blood or serum.

5. A method according to any preceding claim, wherein the body fluid sample is substantially free of cells.

6. A method according to any of the preceding claims, to detect the onset, progression, stabilisation, amelioration and/or remission of prostate cancer.

7. A method according to claim 6, wherein at least two detection and/or quantification steps are provided, spaced apart temporally.

8. A method according to any of the preceding claims, comprising comparing the level of the peptide present in a test sample, with the level in one or more controls.

9. A method of diagnosing prostate cancer comprising quantifying or detecting the amount of at least one homeodomain containing transcription factor, or a fragment thereof, detectable in a body fluid and comparing the amount of said at least one peptide in said test sample with the amount present in a normal control biological sample from a normal, cancer-free, subject, where an increase in the level of the at least one homeodomain containing transcription factor, or a fragment thereof, being found in the test sample is indicative that the disease is either progressing or has initiated, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO. 4, and wherein the fragment has at least 80% sequence homology to SEQ ID NO. 4, and wherein the fragment comprises at least ten consecutive amino acids from said SEQ ID NO: 4.

10. A method according to claim 9, wherein (A) the increase is statistically significant, determined by using a "t-test" providing confidence intervals of at least 80%, and/or (B) wherein (i) an increase between the control and a sample indicative of early stage prostate cancer is around between 110-200%, (ii) an increase between the control and a sample indicative of said late stage prostate cancer is around between 550-620%, (iii) an increase, relative to an early stage sample or control, of around between 100% and 300%, is indicative of progression of the cancer from early to late stage disease.

11. A method of monitoring the efficacy of a treatment for prostate cancer, comprising detecting and/or quantifying at least one homeodomain containing transcription factor, or a fragment thereof, present in a biological sample from a subject, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO. 4, and wherein the fragment has at least 80% sequence homology to SEQ ID NO. 4, and wherein the fragment comprises at least ten consecutive amino acids from said SEQ ID NO: 4.

12. A method according to any of the preceding claims, wherein detection and/or quantification of at least one homeodomain containing transcription factor, or a fragment thereof, detectable in a body fluid is by one or more of the group consisting of: MALDI-TOF, SELDI, via interaction with a ligand or ligands, 1-D or 2-D gel-based analysis systems, Liquid Chromatography combine liquid chromatography and Mass spectrometry techniques including ICAT® (isotype coded affinity tag) or iTRAQ® (isobaric tags for relative absolute quantitation), thin-layer chromatography, and NMR spectroscopy.

13. A method according to any of the preceding claims, wherein detection and/or quantification of the at least one homeodomain containing transcription factor, or a fragment thereof, is by one or more of the group consisting of: sandwich immunoassays, enzyme linked immunosorbent assays (ELISAs), radio-immunoassays (RAI), enzyme immunoassays (EIA), Western Blotting, immunoprecipitation, and particle-based immunoassays.

14. A method according to claim 13, wherein the particle-based immunoassays include using gold, silver, or latex particles, magnetic particles or Q-dots

15. A method according to claim 13 or 14, wherein detection and/or quantification of the at least one homeodomain containing transcription factor, or a fragment thereof, is performed on a microtitre plate, strip format, array or on a chip.

16. A method according to claim 14, wherein detection and/or quantification of the at least one homeodomain containing transcription factor, or a fragment thereof, is by an ELISA comprising antibodies specific for the at least one EN-2 peptide, or a fragment thereof.

17. A method according to claim 16, wherein the antibodies are linked to a reporter.

18. A method according to any of the preceding claims, wherein detection and/or quantification of the at least one EN-2 peptide, or a fragment thereof, is by a biosensor.

19. Use of at least one homeodomain containing transcription factor, or a fragment thereof, detectable in a body fluid, as a biomarker for prostate cancer, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO. 4, and wherein the fragment has at least 80% sequence homology to SEQ ID NO: 4, and wherein the fragment comprises at least ten consecutive amino acids from said SEQ ID NO: 4.

20. Use according to claim 19, wherein said use is in a method selected from the group consisting of: clinical screening, methods of prognosis assessment, monitoring the results of therapy, method to identify patients most likely to respond to a particular therapeutic treatment, and drug screening and development.

21. Use according to claim 19, wherein said patients have previously been diagnosed as negative for cancer using an assay which detects PSA.

22. A method according to claim 1, wherein the detection of the at least one homeodomain containing transcription factor, or fragment thereof, is by indirect detection via interaction with a ligand or ligands, wherein the ligand is an aptamer or oligonucleotide.

23. A method of diagnosing or monitoring the progression of prostate cancer, comprising detecting and/or quantifying RNA encoding a homeodomain containing transcription factor or fragment thereof in a body fluid, wherein the homeodomain containing transcription factor is an EN-2 peptide comprising SEQ ID NO: 4, and wherein the fragment has at least 80% sequence homology to said SEQ ID NO: 4, and wherein the fragment comprises at least ten consecutive amino acids from said SEQ ID NO: 4.

24. A method according to claim 23, wherein the detection and/or quantification of RNA is by quantitative PCR or an oligonucleotide array.

25. A method according to claim 23 or 24, wherein the body fluid is urine.

26. A method according to claim 1 or 9, substantially as hereinbefore described.

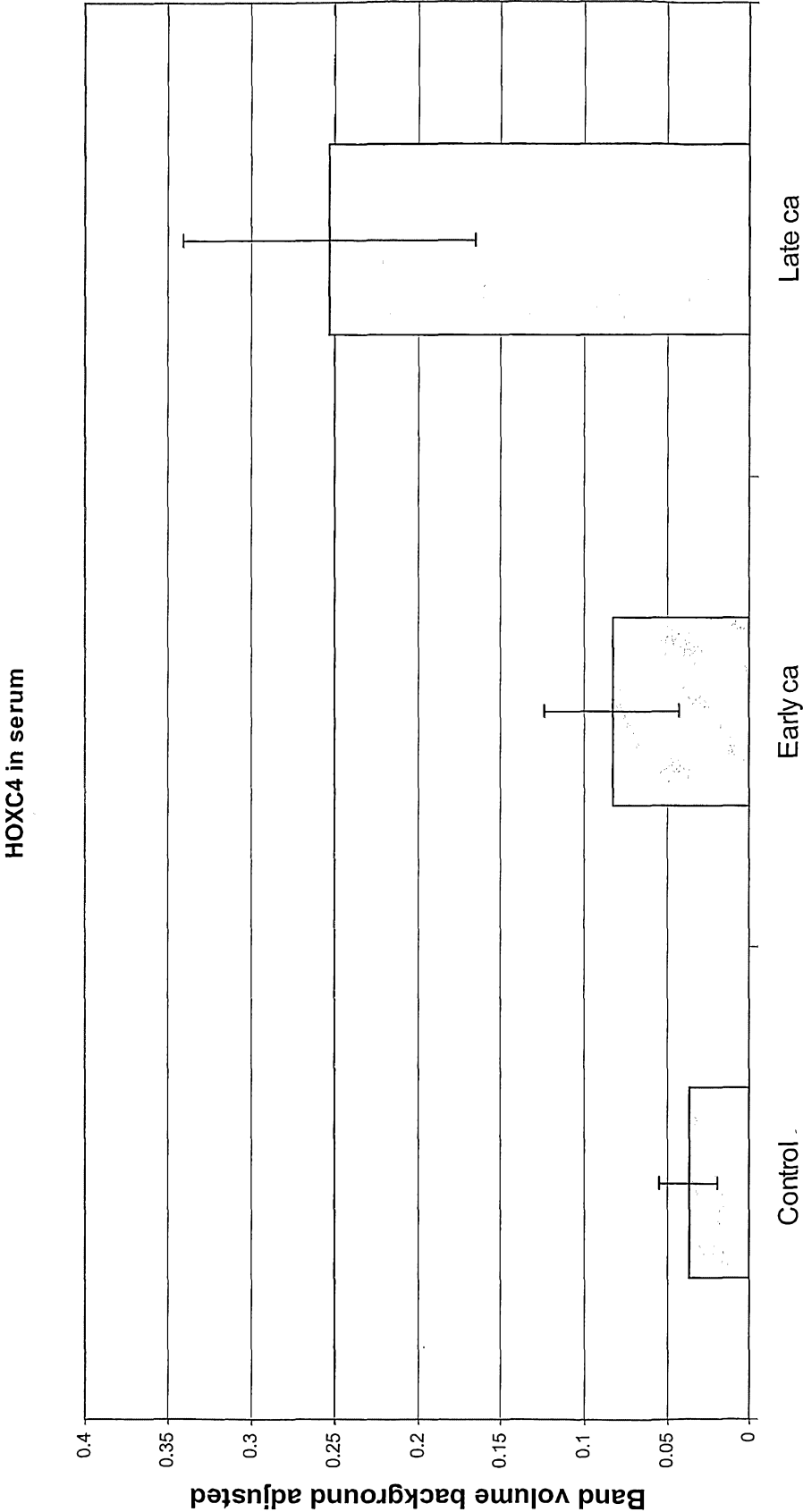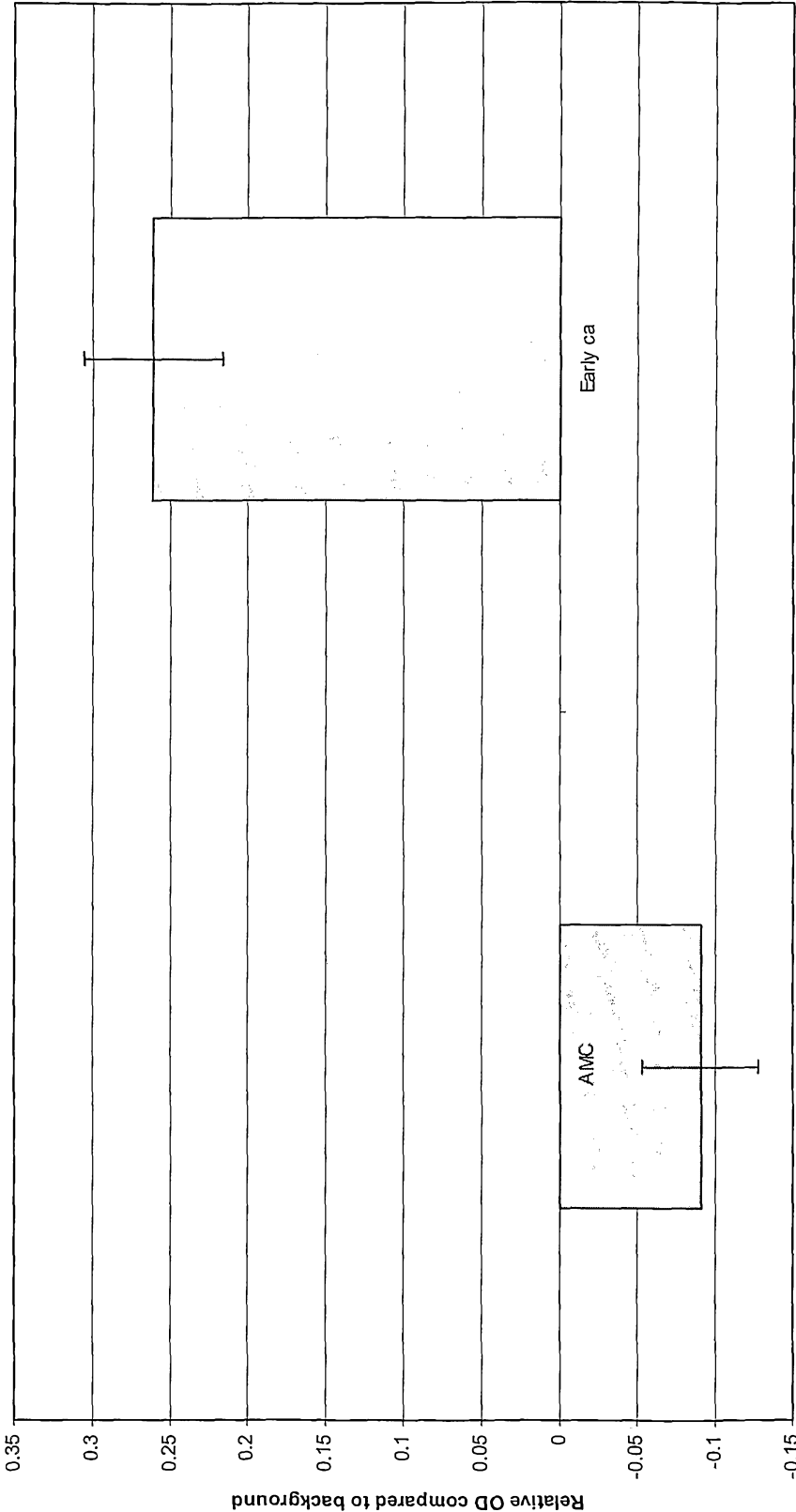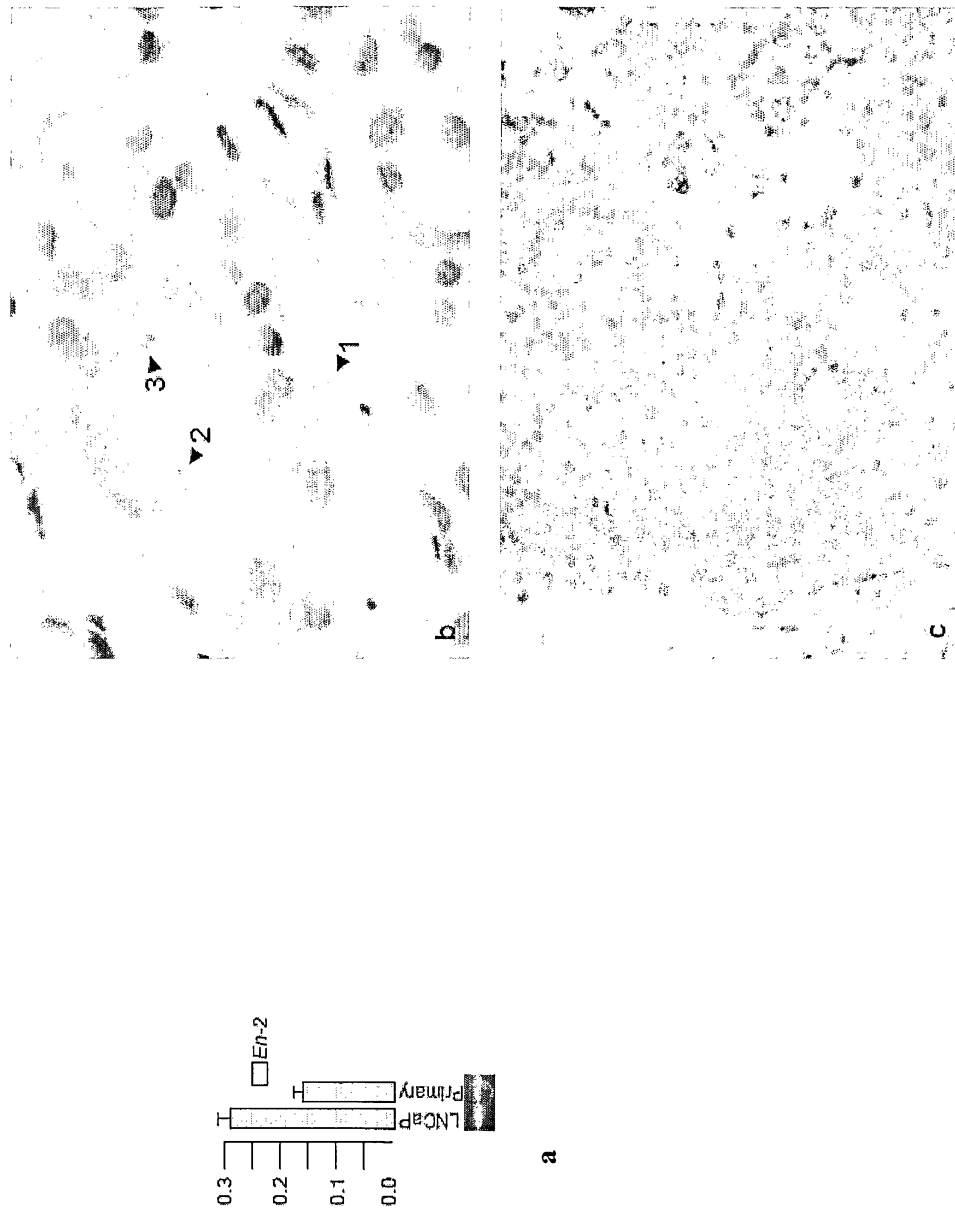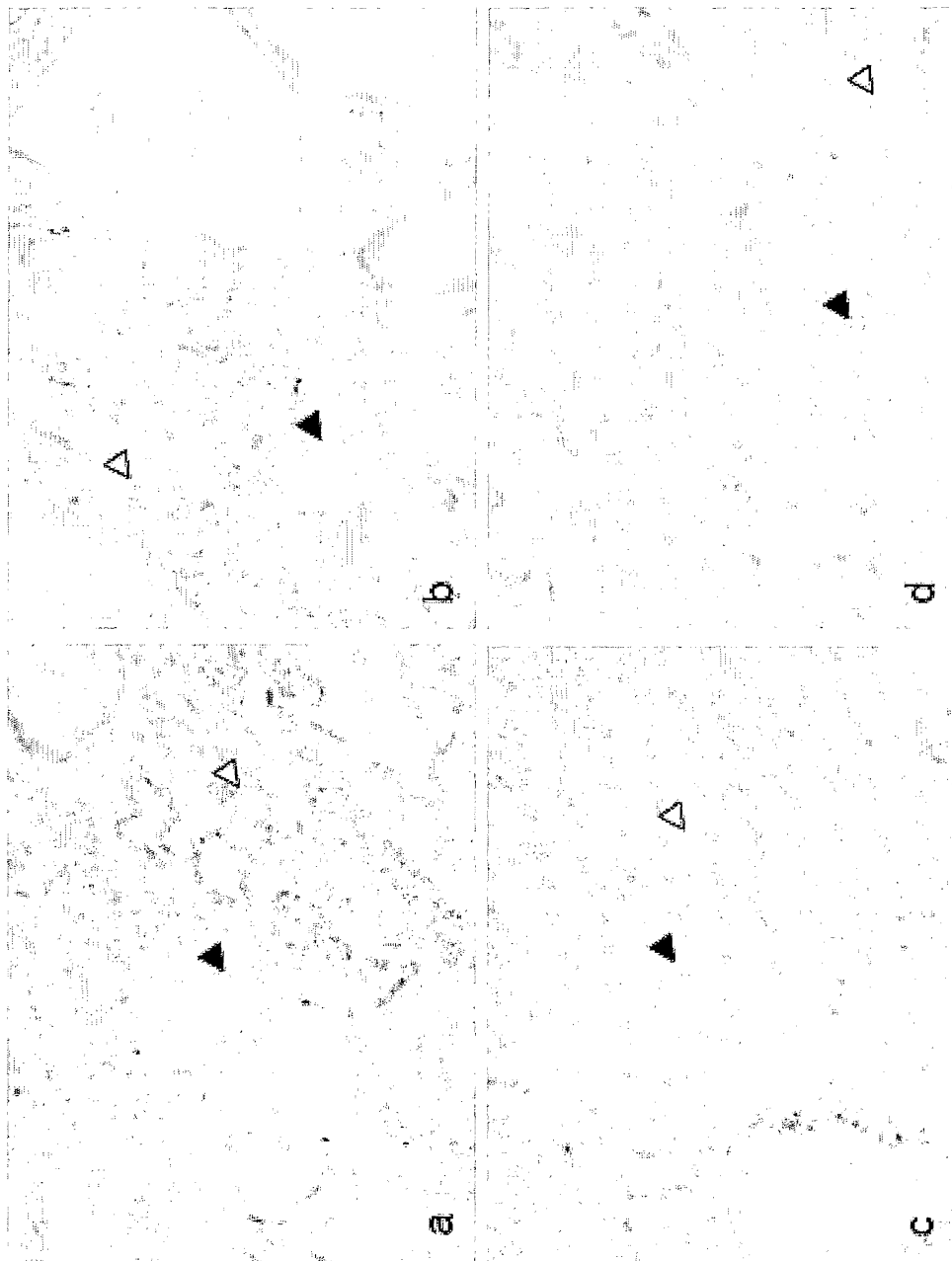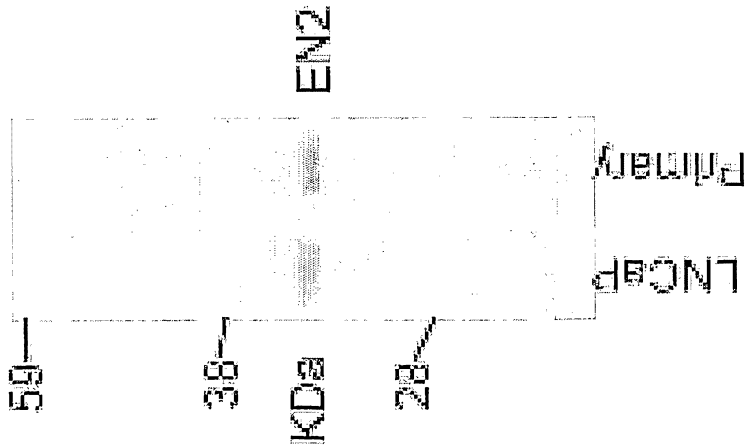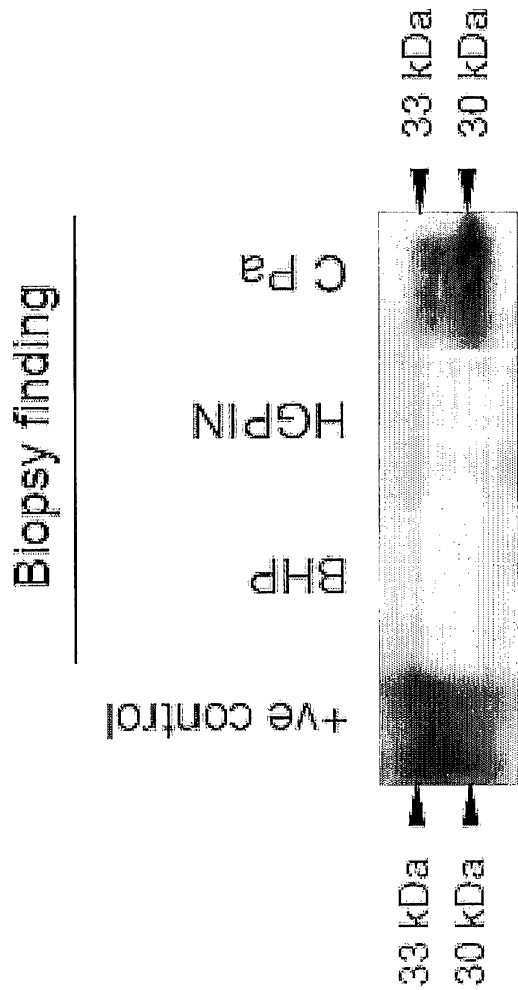5 27. A method according to claim 23, substantially as hereinbefore described.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 6

Figure 5

Figure 7

Figure 8