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**WO 01/12840 A2**

(54) Title: ANTIBODIES AS A CANCER DIAGNOSTIC

(57) Abstract: Method and kits are provided for the detection and diagnosis of metastatic disease. More particularly, the methods and kits employ compounds that can detect EphA2, a specific epithelial cell tyrosine kinase that is overexpressed in metastatic tumor cells. In one embodiment the compound is an antibody capable of binding to an epitope of EphA2.

## ANTIBODIES AS A CANCER DIAGNOSTIC

Field of The Invention

The present invention relates to diagnosis of metastatic disease. More particularly, this invention relates to reagents that can detect a specific epithelial cell tyrosine kinase that is overexpressed in metastatic tumor cells. Most particularly, this invention relates to reagents which bond to an intracellular epitope of the epithelial cell tyrosine kinase, and the use of these reagents for cancer diagnosis.

10 Background And Summary of The Invention

Cancer cell metastasis requires cellular capacity to 1) detach from a primary tumor, 2) migrate and invade through local tissues, 3) translocate to distant sites in the body (via lymph or blood), 4) colonize a foreign site, and 5) grow and survive in this foreign environment. All of these behaviors are linked to cell adhesions. Cell adhesions control the physical interactions of cells with their microenvironment. Cell adhesions also initiate signals that dictate tumor cell growth, death, and differentiation.

Various cancer cells, including breast cancer cells, are known to exhibit altered cell adhesion. As compared to normal breast epithelia, transformed human breast epithelial cells have decreased cell-cell contacts and increased interactions with the surrounding extracellular matrix. These changes facilitate increased detachment and migration of cancer cells away from cell colonies and are directly linked with alteration in tyrosine phosphorylation of cell membrane proteins. Tyrosine phosphorylation is a potent form of cell signal transduction, and alteration in levels of tyrosine phosphorylation is believed to be important for tumor cell invasiveness. Thus, regulation of tyrosine phosphorylation represents a promising target for therapeutic intervention against metastatic cancer. Tyrosine phosphorylation is controlled by cell membrane tyrosine kinases, and increased expression of tyrosine kinases is known to occur in metastatic cancer cells.

30 Identification of increased expression of cell membrane tyrosine kinases would aid in the diagnosis and treatment of metastatic diseases. One such tyrosine kinase is EphA2. A member of the Eph family of tyrosine kinases known as

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Ephrins, EphA2 is a transmembrane receptor tyrosine kinase with a cell-bound ligand. Although cloned a decade ago, see Lindberg, R.A. and Hunter, T., "cDNA Cloning and Characterization of Eck, an Epithelial Cell Receptor Protein-tyrosine Kinase in the Eph/elk Family of Protein Kinases," Mol. Cell. Biol. 10 (12), 6316-6324 (1990),  
5 rather little is known about EphA2 function, largely because EphA2-specific antibodies previously have been difficult to generate.

To facilitate research on EphA2, an improved method for generating a panel of monoclonal antibodies specific for tyrosine phosphorylated proteins has been developed. Using this method, a multiplicity of EphA2 recognizing monoclonal  
10 antibodies has been generated. These antibodies have been used to show that EphA2 is overexpressed in metastatic breast, lung, colon, and prostate cells. Because EphA2 is expressed differently in normal and metastatic cells, EphA2-specific antibodies are useful in the diagnosis of metastatic disease. Antibodies produced by one particular hybridoma recognize an intracellular epitope of EphA2 and have been shown to be  
15 highly specific in binding to EphA2.

Thus, one aspect of this invention is a compound which specifically binds to an intracellular epitope of EphA2. In a preferred embodiment, the compound is an antibody specific for a domain of the EphA2 protein. However, natural or artificial ligands, peptides, anti-sense, ATP analogies, or other small molecules  
20 capable of specifically targeting EphA2 may be employed. A second aspect of this invention is a method for generating antibodies which recognize EphA2 intracellular epitopes. Another aspect of this invention is the use of EphA2-specific antibodies in the diagnosis of metastatic disease. An additional aspect of this invention is a diagnostic reagent specific for detecting EphA2, any fragment thereof, or DNA or  
25 RNA coding for the EphA2 protein. A further aspect of this invention is a kit comprising an antibody capable of specific binding to an epitope of EphA2 and means for detecting antibody-EphA2 binding.

Additional features of the present invention will become apparent to those skilled in the art upon consideration of the following detailed description of preferred embodiments exemplifying the best mode of carrying out the invention as  
30 presently perceived.

### Brief Description of The Drawings

Fig. 1A-C show a series of western blots showing EphA2 expression in cell lines derived from human prostate cells;

Fig. 1A is a western blot showing EphA2 expression in various human prostate cancer cell lines;

Fig. 1B is a western blot showing EphA2 expression in human prostatic epithelial cell line MLC and expression in that cell line after transformation by oncogenic K-Ras or X-irradiation;

Fig. 1C is similar to Fig. 1B, except showing expression in human prostatic epithelial cell line 267B1 and expression in that cell line after transformation by oncogenic K-Ras or X-irradiation;

Fig. 2 is a western blot showing EphA2 expression in various human mammary epithelial cell lines;

Fig. 3A-B shows EphA2 localization in the cell membranes of various mammary epithelial cell lines, as seen by immunofluorescence microscopy;

Fig. 3A shows EphA2 localization in sites of cell adhesion in normal MCF-10A cells; and

Fig. 3B shows EphA2 redistribution in malignant cells.

### 20 Detailed Description of the Invention

Antibodies specific for EphA2 have been isolated through an improved method. The method employed is designed for increased sensitivity and diversity of responding hybridomas. According to this method, tyrosine phosphorylated proteins from Ras-transformed human epithelial cells are isolated by affinity chromatography using existing phosphotyrosine-specific antibodies. The tyrosine phosphorylated proteins are then used as an immunogen for producing monoclonal antibodies. Low-dose amounts of tyrosine phosphorylated proteins are injected proximal to lymph nodes, every other day, over a ten day period (the RIMMS strategy). B cells from engorged lymph nodes are then isolated and fused with a Bcl-2-overexpressing myeloma, to minimize apoptosis after fusion. This method results in increased diversity, specificity, and cost-effectiveness of hybridoma production. The hybridomas are first screened to identify those hybridomas producing antibodies

capable of distinguishing malignant from normal cancer cells. To date, at least 450 such hybridomas have been identified.

Hybridomas which are specific to EphA2 have been selected. Use of the RIMMS strategy has resulted in the production of various monoclonal antibodies  
5 that specifically bind EphA2. Of the first four hybridomas characterized, two recognize independent epitopes on EphA2. The first, D7, recognizes an intracellular epitope. The second, B2D6, binds to an extracellular epitope. D7 has proven to be highly specific for an intracellular epitope of EphA2 and this specificity provides much of the current basis for diagnosis of metastatic tumors.

10 It is known in the art to use antibodies to detect the presence or overexpression of a specific protein. Because EphA2 is overexpressed in metastatic cells, EphA2-specific antibodies of this invention may be used to detect this overexpression and, thus, to detect metastatic disease. Such techniques include but are not limited to western blotting, dot blotting, precipitation, agglutination, ELISA  
15 assays, immunohistochemistry, in situ hybridization, flow cytometry on a variety of tissues or bodily fluids, and a variety of sandwich assays. These techniques are well known in the art. See, for example, U.S. Patent No. 5,876,949, hereby incorporated by reference. When antibodies specific for an intracellular epitope of EphA2 are used, the cells must be lysed and incubated with the antibody. The above techniques  
20 may be performed on whole-cell lysates, or EphA2 may be separated out for testing, such as by immunoprecipitation. The D7 antibodies of this invention are highly specific for an intracellular epitope of EphA2 and have proven to be sensitive to differential expression of EphA2 in metastatic cells. Other techniques, such as immunohistological staining, require whole cells, and may further require cell layers  
25 of a particular cell density. Such tests require an antibody specific for an extracellular epitope of EphA2.

The antibodies of this invention may be used to detect metastatic disease in a wide variety of tissue samples. For instance, research using EphA2-specific antibodies has revealed that altered EphA2 expression occurs in breast,  
30 kidney, prostate, lung, and colon cells, and it is believed that altered EphA2 expression occurs in other types of cell metastasis, particularly epithelial malignancies. EphA2-specific antibodies may be used to detect metastasis in biopsied

tumor tissue. Also, samples of a variety of body fluid samples, such as blood, plasma, spinal fluid, saliva, and urine, can be tested with the antibodies of the present invention. Altered EphA2 expression in these samples indicates the presence of metastatic disease.

5                    Additionally, other antibodies may be used in combination with the antibodies of the present invention to provide further information concerning metastatic disease state. For example, the EphA2 of metastatic cells exhibits altered tyrosine phosphorylation. In normal breast epithelial cells, EphA2 is expressed and is tyrosine phosphorylated. However, in metastatic breast epithelial cells, EphA2 is  
10 overexpressed, and the EphA2 is not tyrosine phosphorylated. Because a test quantifying EphA2 expression sometimes may lead to an ambiguous result, it may be desirable to determine tyrosine phosphorylation, as well as the magnitude of EphA2 expression. Thus, a method of diagnosis using the antibodies of this invention in combination with phosphotyrosine-specific antibodies provides data for determining  
15 the state of metastatic disease.

                    Moreover, the EphA2-specific antibodies of this invention can be exploited to detect changes in EphA2 localization which are associated with metastasis. In normal breast and prostate epithelial cells, EphA2 is enriched in within  
cites of cell adhesion. Conversely, in metastatic prostate cells EphA2 is diffusely  
20 distributed, and in metastatic breast cancer cells EphA2 is redistributed into the membrane ruffles. Techniques such as immunohistological staining or immunofluorescent microscopy are well known in the art and may be used to visualize EphA2 distribution. See, for example, U.S. Patent No. 5,514,554, hereby incorporated by reference. EphA2 expression can be detected by using antibodies  
25 capable of detecting whole EphA2 or fragments of the EphA2 protein. Other methods of detecting altered EphA2 expression include detecting DNA or RNA sequences coding for the EphA2 protein.

                    In order to detect overexpression or altered distribution of EphA2, the EphA2-specific antibodies may be labeled covalently or non-covalently with any of a  
30 number of known detectable labels, such fluorescent, radioactive, or enzymatic substances, as is known in the art. Alternatively, a secondary antibody specific for the

antibodies of this invention is labeled with a known detectable label and used to detect the EphA2-specific antibodies in the above techniques.

Preferred labels include chromogens dyes. Among the most commonly used are 3-amino-9-ethylcarbazole (AEC) and 3,3'-diaminobenzidine tetrahydrochloride (DAB). These can be detected using light microscopy. Also preferred are fluorescent labels. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. Chemiluminescent and bioluminescent compounds such as luminol, isoluminol, therromatic acridinium ester, imidazole, acridinium salt, oxalate ester, luciferin, luciferase, and aequorin also may be used. When the fluorescent-labeled antibody is exposed to light of the proper wavelength, its presence can be detected due to its fluorescence.

Also preferred are radioactive labels. Radioactive isotopes which are particularly useful for labeling the antibodies of the present invention include  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ , and  $^{14}\text{C}$ . The radioactive isotope can be detected by such means as the use of a gamma counter, a scintillation counter, or by autoradiography.

Another method in which the antibodies can be detectably labeled is by linking the antibodies to an enzyme and subsequently using the antibodies in an enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA). The enzyme, when subsequently exposed to its substrate, reacts with the substrate and generates a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric, or visual means. Enzymes which can be used to detectably label antibodies include, but are not limited to malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholinesterase. Other methods of labeling and detecting antibodies are known in the art and are within the scope of this invention.

### Example 1

The antibodies produced by the D7 hybridoma are used to detect differential expression of EphA2 between normal prostate epithelial cells and metastatic cells. Fig. 1 shows EphA2 expression in various human prostate cell lines. Referring first to Fig. 1A, three metastatic cell lines, LNCAP, DU145, and PC3, are tested for levels of EphA2 expression. It is known that, of these three cell lines, LNCAP is the least invasive, DU145 is somewhat more invasive, and PC3 is the most invasive. EphA2 expression is determined by western blotting with D7 antibodies. As can be seen in Fig. 1A, EphA2 expression positively correlates with invasiveness.

In Fig. 1B, D7 antibodies are used to test EphA2 expression in normal MLC cells as compared to expression in transformed cells. Normal MLC cells, MLC cells which have been transformed by K-Ras, and MLC cells which have been transformed by X-irradiation are studied. As can be seen in Fig. 1B, EphA2 is overexpressed in both of the transformed cell lines. Fig. 1C shows results similar to Fig. 1B, except the normal cell line is 267B1. As with Fig. 1B, Fig. 1C shows that EphA2 is overexpressed in the transformed cells. In sum, Fig. 1 demonstrates that EphA2-specific antibodies detect changes in metastatic cells, and that tests using these antibodies indicate the level of metastatic invasiveness.

### Example 2

EphA2 antibodies are used to detect altered EphA2 expression in metastatic mammary cells. EphA2 is expressed in normal mammary epithelial cells. Fig. 2 illustrates altered EphA2 expression in mammary tumor cell lines. As can be seen in Fig. 2, western blots from whole cell lysates using D7 antibodies reveal that EphA2 expression is completely absent in cells derived from non-metastatic breast tumors (ZR75-1, BT474, SKBR3, MDA-MB-435). By contrast, EphA2 is overexpressed in metastatic breast cancer cell lines (MDA-MB-435, MDA-MB-231). Thus, EphA2 antibodies detect altered EphA2 expression in breast cancer cells, which can be used to diagnose metastasis. Moreover, in non-metastatic breast epithelial cells, loss of EphA2 occurs early in the disease, and testing with EphA2-specific antibodies provide information relevant to invasiveness even when other known

markers remain normal. Thus, D7 antibodies are useful as a diagnostic, even in early stages of disease.

### Example 3

5 EphA2 antibodies in combination with other antibodies are used to detect further alterations in EphA2 expression. As discussed above in Example 2, western blots using D7 can distinguish between non-metastatic and metastatic tumors, with non-metastatic tumors failing to express EphA2, and metastatic cells overexpressing EphA2. However, different results are found when tyrosine  
10 phosphorylation is studied. Using a phosphotyrosine-specific antibody, it has been found that EphA2 is phosphorylated in normal cells, but it is not phosphorylated in metastatic cells. Thus, while EphA2 specific antibodies can qualitatively detect a difference between metastatic and non-metastatic mammary tumor cells, diagnostics incorporating both an EphA2-specific antibody and a phosphotyrosine-specific  
15 antibody provides a sensitive test for distinguishing between normal, non-metastatic, and metastatic mammary cells.

### Example 4

Immunofluorescently labeled EphA2-specific antibodies detect  
20 redistribution of EphA2 expression in transformed cells. The EphA2-specific antibodies used in this example are produced by a cell line known as B2D6, and these antibodies are specific for an extracellular epitope of EphA2. As seen in Fig. 3A, immunofluorescence with B2D6 demonstrates that EphA2 is found within sites of cell-cell contact in normal cells. However, in transformed cells, shown in Fig. 3B,  
25 EphA2 is redistributed. Furthermore, in metastatic cells EphA2 is found in the membrane ruffles. Similarly, in normal prostate epithelial cells, EphA2 is found within sites of cell-cell adhesion, but in metastatic prostate epithelial cells, EphA2 is overexpressed and the expression is diffusely distributed. Therefore, immunofluorescence using EphA2-specific antibodies provides an additional means  
30 for diagnosing the transformation and metastatic state of tumor cells.

As shown in Examples 1-4, overexpression, redistribution, and phosphorylation of EphA2 in metastatic cells provide various bases for diagnosis of metastatic tumors using EphA2-specific antibodies. Immunohistochemistry or Western blotting may be used to monitor the change of EphA2 expression in biopsied  
5 samples of patient breast tissue, prostate tissue, or tissue from other tumors. Additionally, D7 and other EphA2-specific antibodies can be used to monitor plasma, urine, and other body fluids to detect altered expression of EphA2, which would signal metastasis. Detection of altered tyrosine phosphorylation of EphA2 in combination with information concerning an alteration of EphA2 expression further  
10 aids in diagnosis of metastatic disease.

Although the invention has been described in detail with reference to preferred embodiments, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.

CLAIMS

1. A method for detecting the presence of metastatic cells in a cell population comprising the steps of  
5 lysing at least a portion of the cell population,  
incubating the lysed cells with a reagent capable of specific binding to an epitope of EphA2 to allow antibody binding to said epitope, and  
detecting compound-epitope binding.
2. The method of claim 1 wherein the reagent is an antibody.
- 10 3. The method of claim 2 wherein the epitope of EphA2 is an intracellular epitope of EphA2.
4. The method of claim 3 wherein the antibody is produced by hybridoma cell line D7.
5. The method of claim 2 wherein the antibody is labeled with a  
15 detectable label, and the detecting step includes detecting the label.
6. The method of claim 5 wherein the antibody is labeled with a fluorescent label and the detecting step comprises detecting the fluorescent label.
7. The method of claim 5 wherein the antibody is labeled with a radioactive label and the detecting step comprises detecting the radioactive label.
- 20 8. The method of claim 1 wherein the cell population comprises cells from a breast or prostate tissue biopsy.
9. The method of claim 1 wherein the cell population is harvested from a body fluid selected from the group consisting of blood, plasma, spinal fluid, saliva, and urine.
- 25 10. The method of claim 9 wherein the detecting step includes a diagnostic method selected from the group consisting of ELISA assays and flow cytometry.
11. The method of claim 1 wherein the incubating and detecting steps comprise western blotting methodology.
- 30 12. The method of claim 11 further comprising the steps of providing a second antibody having phosphotyrosine specificity, and western blotting with the second antibody.

13. The method of claim 1 wherein the metastatic cells are selected from the group consisting of breast, prostate, lung, and colon cancers.
14. A method of producing an antibody which specifically binds to an intracellular epitope of EphA2 comprising the steps of
- 5 injecting tyrosine phosphorylated proteins into lymph nodes of a mammal,
- harvesting lymph node cells from the mammal,
- fusing lymph node cells with myeloma cells to form hybridomas,
- selecting at least one hybridoma producing an antibody which binds to
- 10 the intracellular epitope of EphA2,
- isolating the antibody.
15. The method of claim 14 wherein the antibody recognizes an antigen also recognized by the monoclonal antibody D7.
16. The method of claim 14 wherein the tyrosine phosphorylated
- 15 proteins are EphA2.
17. The antibody produced by the method of claim 14.
18. An antibody which specifically binds to an intracellular epitope of EphA2.
19. The antibody of claim 18 bound to a detectable labeled.
20. The antibody of claim 19 which is produced by hybridoma cell
- 20 line D7.
21. A method for detecting the presence of metastatic cells in a cell population comprising the steps of
- incubating the cells with a reagent capable of specific binding to a
- 25 compound associated with EphA2 expression, and
- detecting reagent-compound binding.
22. The method of claim 21 wherein the reagent is an antibody.
23. The method of claim 21 wherein the compound is selected from the group consisting of EphA2, a fragment of EphA2, DNA coding for the EphA2
- 30 protein, and RNA coding for the EphA2 protein.
24. The method of claim 21 further comprising the step of fixing the cells on a slide, and the detecting step comprises immunofluorescence staining.

25. A kit for detecting the presence of metastatic cells in a cell population comprising  
an antibody capable of specific binding to an epitope of EphA2, and means for detecting antibody-epitope binding.
- 5 26. The kit of claim 25 wherein the means for detecting antibody-epitope binding is a label bound to the antibody.
27. The kit of claim 25 further comprising an antibody having phosphotyrosine specificity.



FIG. 1A

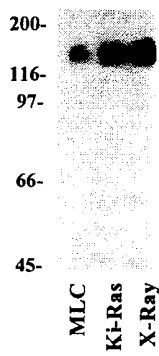


FIG. 1B

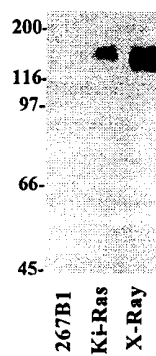


FIG. 1C

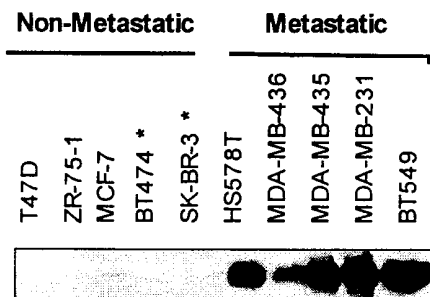
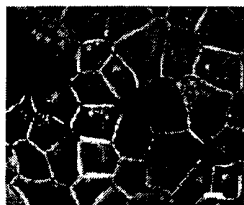


Fig. 2



**FIG. 3A**




**FIG.3B**

**INDICATIONS RELATING TO A DEPOSITED MICROORGANISM**

(PCT Rule 13bis)

<p><b>A.</b> The indications made below relate to the microorganism referred to in the description  on page <u>4</u> , line <u>7</u> .</p>	
<p><b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span></p>	
<p>Name of depositary institution  American Type Culture Collection</p>	
<p>Address of depositary institution <i>(including postal code and country)</i>  10801 University Boulevard  Manassas VA 20110-2209  United States of America</p>	
<p>Date of deposit  8 December 2000 (08.12.00)</p>	<p>Accession Number  PTA 2754</p>
<p><b>C. ADDITIONAL INDICATIONS</b> <i>(leave blank if not applicable)</i> <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span></p>	
<p>Murine hybridoma B2D6</p>	
<p><b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> <i>(if the indications are not for all designated States)</i></p>	
<p><b>E. SEPARATE FURNISHING OF INDICATIONS</b> <i>(leave blank if not applicable)</i></p>	
<p>The indications listed below will be submitted to the International Bureau later <i>(specify the general nature of the indications e.g., "Accession Number of Deposit")</i></p>	

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(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>4</u> , line <u>6</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 10801 University Boulevard Manassas VA 20110-2209 United States of America	
Date of deposit 8 December 2000 (08.12.00)	Accession Number PTA 2755
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
Murine hybridoma D7	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
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