The present invention provides methods for inhibiting cancerous growth of a cell using an antibody or an antigen-binding portion thereof which binds to an epitope of EphB4 polypeptide. Purified antibodies of EphB4 are also provided. The invention also provides methods for preventing or treating cancer. The invention also relates to methods of identifying agents that can inhibit cancerous growth of a cell.
METHODS FOR REGULATING CANCER

The present invention provides methods for inhibiting cancerous growth of a cell using an antibody or an antigen-binding portion thereof which binds to an epitope of EphB4 polypeptide. Purified antibodies of EphB4 are also provided. The invention also provides methods for preventing or treating cancer. The invention also relates to methods of identifying agents that can inhibit cancerous growth of a cell.
Methods For Regulating Cancer

Field of the invention:

The present invention relates to methods for regulating cancer. In particular, the invention relates to methods for inhibiting cancerous growth of a cell. The invention also provides methods for preventing or treating cancer. The invention also relates to methods of identifying agents that can inhibit cancerous growth of a cell.

Background of the invention:

Cancer describes a range of diseases, which result from dysregulated growth of cells of the body. Malignant cancers may develop from this dysregulated growth and subsequently spread around the body via the bloodstream or the lymphatic system, a process known as metastasis. Malignant tumours of epithelial tissues are the most common form of cancer and are responsible for the majority of cancer-related deaths in western industrialised countries. According to the Australian Institute of Health and Welfare (AIHW), on average one in three men and one in four women will develop cancer before the age of 75 years (1). In men the most common cancers are prostate, bowel and lung and in women, breast, bowel and melanoma. Identification of genes expressed specifically in tumour tissues and not in normal tissues, and analysis of their functions are useful for identifying new targets for cancer therapy.

Several genes have been implicated in various cancers. For instance, oncogenes are known to code for receptors for cellular growth factor such as epidermal growth factor. The ras gene is an oncogene that is believed to be responsible for up to 90% of all human pancreatic cancer, 50% of human colon cancers, 40% of lung cancers, and 30% of leukemias. Mutated oncogenes can become cancer-causing genes. Such mutated oncogenes code for proteins such as protein kinases and protein phosphorylating enzymes that trigger uncontrolled cell growth. EphB4 is a recently identified member of the largest known family of receptor protein tyrosine kinases. Eph receptor family members have been identified to be involved in many cellular processes including neural development, angiogenesis and vascular network assembly (2-5). As a result of interactions with their ligands, the ephrins, they mediate contact-dependent cell
interactions, which regulate cell functions such as contact inhibition, cytoskeletal organisation and cell migration (6, 7).

Although a number of anti-cancer agents including growth inhibitory molecules such as cytotoxic compounds have been developed in an attempt to treat cancer, there still remains a need for providing effective methods for regulating cancer.

**Summary of the invention:**

The present invention is based on the surprising finding that an antibody that can bind to a particular region of the EphB4 protein can advantageously inhibit cancerous growth in a cancer cell by causing cell death of the cancer cell.

Therefore, in a first aspect the present invention provides a method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto.

Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

In a second aspect the present invention also provides a method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding
portion thereof binds to an epitope located within residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

In a third aspect the present invention provides a method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

In another aspect of the invention there is provided a method of identifying an agent which inhibits cancerous growth of a cell, the method comprises assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the method comprises assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the method comprises assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the method comprises assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at
least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

The present invention also provides an agent identified by the method described above.

In a further aspect of the invention there is provided a purified EphB4 antibody which binds to a polypeptide having a sequence at least 85% identical to residues 201 to 245 of EphB4 (SEQ ID NO: 1), preferably at least 90% identical to residues 201 to 245 of EphB4 (SEQ ID NO: 1). Preferably, the purified EphB4 antibody binds to a polypeptide having a sequence at least 85% identical to residues 220 to 244 of EphB4 (SEQ ID NO: 1), preferably at least 90% identical to residues 220 to 244 of EphB4 (SEQ ID NO: 1). The purified EphB4 antibody preferably binds to a polypeptide having a sequence at least 85% identical to residues 220 to 230 of EphB4 (SEQ ID NO: 1), preferably at least 90% identical to residues 220 to 230 of EphB4 (SEQ ID NO: 1). Most preferably, the present invention provides a purified EphB4 antibody which binds to an epitope located in residues 200 to 400 of EphB4 (SEQ ID NO: 1). The purified EphB4 antibody according to the present invention preferably binds to a polypeptide having a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1). Preferably, the purified EphB4 antibody is a monoclonal antibody.

**Brief description of the figures:**

Figure 1 shows immunohistochemical localisation of EphB4 expression in three different colon cancers and matched normal mucosa using the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). The dark stain from the biotinylated secondary antibody indicates the EphB4 protein. Nuclei are stained with Harris haematoxylin. High power (100X) magnification images of three different adenocarcinomas (well differentiated, moderately well differentiated and poorly differentiated) and their matched normal mucosa are shown. Strong staining of the tumour tissue and very weak, diffuse staining of normal tissue was evident for each sample set. There was no cross-reactivity with the secondary antibody alone (result not shown).
Figure 2 shows relative RT-PCR comparing expression of EphB4 (1187 bp) and internal 18S rRNA (489 bp) in five tumour (T) / normal (N) pairs. LM-Liver metastasis and NL-normal liver from patients 5, C1 – colon cancer cell line LIM2405, C2 – colon cancer cell line SW480, RT-RT negative control, P – PCR negative control, M – pUC19 / HpaII marker.

Figure 3 shows a confluent monolayer of cells in 2 ml DMEM is treated with a 1/500 dilution of the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) (0.4 ng/μl final concentration). Incubation of cells with the antibody causes a visual response between 24 and 48 h. The cells lift off the bottom of the culture vessel in a fragile sheet which breaks up easily on gentle agitation. The change in colour of the media is due to a 0.5 pH unit change attributed to the leakage of cellular contents from dead cells.

Figure 4 shows a graph showing effect of increased doses of an EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) on growth of MCF-7 cells in vitro after 48 hours.

Figure 5 shows a trypan blue exclusion assay to determine dose dependency of an EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). The number of viable cells (x100000) (Y-axis) decreases after 48h exposure to three different dilutions of the EphB4 antibody (X-axis). There were no viable cells remaining after 72h with the addition of 1μg / ml and after 48h with the addition of 2 μg / ml.

Figure 6 shows a Caspase-3 assay. The relative amount of caspase-3 activity per 100000 cells (Y-axis) increases after time for all dilutions of an EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) (X-axis). Because there were no viable cells counted for 1/200 dilution (1 μg/ml) after 72h and for the 1/100 (2 μg/ml) dilution after 48h, caspase-3 activity was not measured.

Figure 7 shows a graph showing percentage viability of breast cancer cells after 65 h treatment with five different EphB4 antibodies: (1) a EphB4 polyclonal antibody
(Swiss) directed to amino acid residues 825 to 991 of the carboxy terminus of mouse EphB4 (gift from Dr Andrew Ziemiecki, University of Bern), (2) a polyclonal N-terminal EphB4 antibody (N-19 Santa Cruz Biotechnology) directed to the N-terminal first 19 amino acids of the EphB4 amino acid sequence which is likely to be amino acids residues 16 to 34 of the mature EphB4 (SEQ ID NO:1), (3) a polyclonal EphB4 C-terminal antibody (C-16 Santa Cruz Biotechnology) directed to the carboxy-terminal corresponding to tyrosine kinase domain consisting of amino acid residues 615 to 874 of EphB4 (SEQ ID NO:1), (4) a EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) and (5) EphB4 polyclonal antibody (H-200 (old) -Santa Cruz Biotechnology -Lot number B141 batch) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). Cells were treated with 1/100 dilution of stock antibody (200 μg/ml), then stained with trypan blue (stains dead cells). Ratios of unstained (viable) to stained (unviable) were calculated for four different aliquots of each treatment. Control – no antibody added. CLM – complement limited medium. FCS – 10% Fetal calf serum added to medium. Complement does not play a role in the cell death effect of the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). This is demonstrated by the comparison of percentage viability after antibody addition to cells grown in medium with normal protein activity (FCS experiment) with cells grown in medium in which complement proteins were inactivated by heating to 55°C for 30 mins (CLM Experiment).

**Figure 8** shows a western analysis of normal human tissues as indicated and a representative colon tumour (Tumour). (A) EphB4 protein was identified using a EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). Predicted wildtype protein is 120 kDa and is indicated by the arrow. (B) Coomassie blue stained duplicate gel. Sizes of molecular weight marker are as indicated.

**Figure 9** shows a schematic diagram comparing the domains of the wild-type EphB4 receptor with the predicted structure of the slice variants EphB4v1 and EphB4v2. Deleted regions are indicated by *.
Figure 10 shows an expression of EphB4 gene and PBGD (housekeeping control) in breast cancer cell lines MDA-MB-231 (231), MDA-MB-468 (468), Hs578t, MCF7 and T47D either treated with 1/100 dilution of the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) for 65 h (Treated) or with no treatment (control). RT-ve is the reverse transcription reagents only only control. Marker 1 – Spp1/EcoR1 and Marker 2 – pUC19/Hpa11.

Figure 11 shows a sequences of the six overlapping peptides [shown as SEQ ID NO: 2 (Peptide 1) to SEQ ID NO:7 (Peptide 6)] designed to span the first 125 amino acids of the target EphB4 sequence (shown in bold). The numbers refer to the position of the amino acids in the mature EphB4 protein (SEQ ID NO:1 shown in Figure 18).

Figure 12 shows a trypan blue exclusion assay comparing viability in control cells (untreated), cells with the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) alone (Ab only) or treated with both antibody and peptide cocktail (Ab + all peptides). Tests were performed in duplicate.

Figure 13 shows an assay comparing relative levels of caspase-3 activity in control cells (untreated), cells with the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) alone, (Ab only) or treated with both antibody and peptide cocktail (Ab + all peptides). Tests were performed in duplicate.

Figure 14 shows results of trypan blue exclusion assay performed 48 h after treatment on confluent SW480 monolayers with a 1/500 dilution of the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) with or without 5 μl of peptides as indicated. Peptides 1 and 2 appeared to rescue ~50% of cells from Ab-mediated cell death.

Figure 15 shows an increase in the concentration of Peptide 1 (SEQ ID NO:2) or Peptide 2 (SEQ ID NO: 3) (to 10 μl) was able to fully rescue cells from the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the
extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) mediated cell death and was equal in effect to a combination of Peptides 1 and 2 (5 \mu l of each).

Figure 16 shows a sequences of the two overlapping peptides [shown as SEQ ID NO:2 (Peptide 1) and SEQ ID NO:3 (Peptide 2)] that were able to block the function of the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) on cells in culture and sequences of three peptides [shown as SEQ ID NO:8 (Peptide 7) to SEQ ID NO:10 (Peptide 9)] designed about the core sequence GSCVV for further narrowing of the reactive sequence. The numbers refer to the position of the amino acids in the mature EphB4 protein (sequence shown in bold font).

Figure 17 shows Peptide 7 (SEQ ID NO: 8) was able to fully rescue cells from Ab-mediated cell death and was equal in effect to Peptide 2 (SEQ ID NO: 3). One microlitre of the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) (0.2\mu g/ml) and 10 \mu l of a 10 mg/ml stock of peptide were pre-incubated together on ice for 2 h before addition to a confluent monolayer of cells in a 24-well microtitre plate with 500 \mu l of DMEM.

Figure 18 shows the amino acid sequence of SEQ ID NO:1. SEQ ID NO:1 is the amino acid sequence of mature Homo sapiens Ephrin type-B receptor 4 (EphB4).

Figure 19. shows a model of the EphB4 receptor with domain regions to which the different polyclonal antibodies (N-19, H-200 and C-16) have been targeted indicated by black brackets. The globular domain (ephrin receptor ligand binding domain) corresponds to residues 29 to 197 of EphB4 (SEQ ID NO:1). The cysteine-rich region (Giardia variant-specific surface protein) corresponds to residues 255 to 313 of EphB4 (SEQ ID NO:1). The fibronectin type III domain 1 corresponds to residues 324 to 414 of EphB4 (SEQ ID NO:1). The fibronectin type III domain corresponds to residues 437 to 517 of EphB4 (SEQ ID NO:1). The transmembrane domain corresponds to residues 540 to 560 of EphB4 (SEQ ID NO:1). The tyrosine kinase domain corresponds to residues 615 to 874 of EphB4 (SEQ ID NO:1). The SAM (sterile alpha motif) domain corresponds to residues 904 to 971 of EphB4 (SEQ ID NO:1). The PDZ domain corresponds to residues 985 to 987 of EphB4 (SEQ ID NO:1).
The placements of these domains relative to the EphB4 amino acid sequence is based on information taken from the most recent report from NCBI Accession number NP_004435. The N-19 Antibody maps to the N-terminal first 19 amino acids of the sequence which is likely to be amino acids residues 16 to 34 of the mature EphB4 (SEQ ID NO:1). The C-16 antibody is directed to the tyrosine kinase domain. The H-200 antibody is specifically directed to residues 201 to 400 of EphB4 (SEQ ID NO:1) in the extracellular domain spanning the cysteine rich region and the fibronecton domain.

**Figure 20** shows a sequence of a Peptide 11 (SEQ ID NO: 12) designed to include the proposed epitope sequence and a Peptide 10 (SEQ ID NO:11) in which the amino acid Aspartate (D) which carries a charge in this wild-type sequence is substituted with an uncharged amino acid with a similar side chain structure Asparagine (N). The numbers and the sequence in bold font refer to the position of the amino acids in the mature EphB4 protein.

**Detailed description of the invention:**

In a first aspect the present invention provides a method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

The antibody or an antigen-binding portion thereof preferably specifically binds to a polypeptide having a sequence consisting of residues 200 to 400 of EphB4 (SEQ ID NO:1). Preferably, the antibody or antigen-binding portion specifically binds to a polypeptide having a sequence consisting of residues 201 to 245 of EphB4 (SEQ ID NO:
1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion specifically binds to a polypeptide having a sequence consisting of residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or antigen-binding portion thereof specifically binds to a polypeptide having a sequence consisting of residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1). Most preferably, the antibody or an antigen-binding portion thereof is a polyclonal or monoclonal antibody. The method preferably results in the death of the cell.

The antibody or antigen-binding portion thereof preferably specifically binds to a polypeptide having a sequence at least 85%, preferably at least 90% identical to sequence selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO:1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1). A polypeptide having a sequence at least 85%, preferably at least 90% identical to residues 200 to 400 of EphB4 (SEQ ID NO:1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) or residues 220 to 230 of EphB4 (SEQ ID NO: 1), preferably includes polypeptide variants having at least one substitution, deletion or addition of particular amino acid(s). Such polypeptide variants are also suitable for the present methods, particularly if they retain antigenic properties. For instance, the polypeptide variants can be designed to retain antigenic properties and to improve polypeptide production and/or solubility.

For example, antigenic prediction programs suggest that the charged amino acid Asp (D) may also be important to the epitope function as it is the only charged residue in the sequence. Peptide 11 consisting of amino acid residues 220 to 244 of EphB4 protein (SEQ ID NO:1) was designed as indicated in Figure 20. Peptide 10 with a substitution of Asn (N) at residue 226 of EphB4 protein (SEQ ID NO:1) was also designed as indicated in Figure 20). The amino acid sequence of Peptide 10 and Peptide 11 is as follows:

Peptide 10    SEQ ID NO:11:    AGSCVNAVPAAGPSLYCREDGQ
Peptide 11    SEQ ID NO:12:    AGSCVVDAVPAAGPSLYCREDGQ
The side chains of Asp and Asn are very similar – the hydroxyl group of Asp is an amine in Asn and changes it from being a negatively charged amino acid to a neutral one.

In a second aspect the present invention also provides a method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

In a third aspect the present invention provides a method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).
The cancer is preferably selected from the group consisting of breast cancer, prostate cancer, bowel cancer, bladder cancer, colon cancer, ovarian cancer, lung cancer, melanoma, lymphoma and leukemia. The method preferably results in the death of a cancer cell in the subject.

In another aspect of the invention there is provided a method of identifying an agent which inhibits cancerous growth of a cell, the method comprising assessing the ability of the agent to bind to an EphB4 polypeptide within the region of 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto.

In a preferred embodiment of the invention, the agent binds to an epitope contained within residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the agent binds to an epitope contained within residues 201 to 245 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the agent binds to an epitope contained within residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO:1).

The present invention also provides an agent identified by the method described above.

In the present specification the term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments. The term "epitope" refers to an epitope region of a polypeptide that is recognized by an antibody or an antigen binding portion thereof.

Antibodies refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (HCVIR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of...
three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein refers to one or more fragments of an antibody that retains the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full length antibody.

Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody; (v) a dAb fragment (8) which consists of a VH domain, or a VL domain (9); and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv) (10), (11). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies or triabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (12, 13). Preferably, the antibody is EphB4 (H-200) rabbit polyclonal Ig G antibody, Santa Cruz Biotechnology, Santa Cruz, California.
More preferably, the antibody is a monoclonal antibody or fragment thereof and, particularly, is selected from monoclonal antibodies or fragments thereof which bind to an epitope within residues 200 to 400 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the monoclonal antibodies or fragments thereof bind to an epitope within residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the monoclonal antibodies or fragments thereof bind to an epitope within residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the monoclonal antibodies or fragments thereof bind to an epitope within residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

In a further aspect of the invention there is provided a purified EphB4 antibody which binds to a polypeptide having a sequence at least 85% identical to residues 201 to 245 of EphB4 (SEQ ID NO: 1), preferably at least 90% identical to residues 201 to 245 of EphB4 (SEQ ID NO: 1). Preferably, the purified EphB4 antibody binds to a polypeptide having a sequence at least 85% identical to residues 220 to 244 of EphB4 (SEQ ID NO: 1), preferably at least 90% identical to residues 220 to 244 of EphB4 (SEQ ID NO: 1). The purified EphB4 antibody preferably binds to a polypeptide having a sequence at least 85% identical to residues 220 to 230 of EphB4 (SEQ ID NO: 1), preferably at least 90% identical to residues 220 to 230 of EphB4 (SEQ ID NO: 1). More preferably, the present invention provides a purified EphB4 antibody which binds to an epitope located in residues 200 to 400 of EphB4 (SEQ ID NO: 1). The purified EphB4 antibody according to the present invention preferably binds to a polypeptide having a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1). More preferably, the purified EphB4 antibody is a monoclonal antibody.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different
antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method, isolated from phage antibody libraries, or may be made by recombinant DNA methods. Such techniques include, but are not restricted to, the hybridoma technique (14), the trioma technique, the human B-cell hybridoma technique (15), and the EBV hybridoma technique to produce human monoclonal antibodies (16). In addition, humanised monoclonal antibodies can be generated according to methods described in US patent 6,090,382 of which the entire description and references cited therein are incorporated herein. The document provides suitable host cells for expressing recombinant human antibodies and methods of synthesising the recombinant human antibodies. Furthermore, suitable human antibodies may be produced using transgenic animals using for example techniques described in Oncology 29 (Supp 4) 47-50 (2002). The antibodies of the present invention may also be obtained from commercial sources.

Various procedures known in the art may also be used for the production of polyclonal antibodies which can bind to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. For production of the antibodies, various host animals can be immunized by injection with a EphB4 protein or a EphB4 polypeptide fragment bound to a suitable carrier. Suitable carriers can include, but are not limited to, BSA (bovine serum albumin), KLH (keyhole limpet hemocyanin), OVA (ovalbumin), THY (Thyroglobulin) and RSA (rabbit serum albumin). The host animal is preferably immunized with a EphB4 polypeptide comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the host animals can be immunized by injection with a EphB4 protein or a polypeptide comprising residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the host animals can be immunized by injection with a EphB4 protein or a polypeptide comprising residues 220 to 244 of
EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the host animals can be immunized by injection with a EphB4 protein or a polypeptide comprising residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

Suitable host animals include, but are not limited to, rabbits, mice, rats, etc. Various adjuvants can be used to increase the immunological response, depending on the host species, and include, but are not limited to, Freud's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium parvum*. Antibodies and antibody fragments may be produced in large amounts by standard techniques (eg in either tissue culture or serum free using a fermenter) and purified using affinity columns such as protein A (eg for murine Mabs), Protein G (eg for rat Mabs) or MEP HYPERCEL (eg for IgM and IgG Mabs).

Suitable antibodies may include antibody fragments that include an antigen-binding portion that can bind to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. The antigen-binding portion of an antibody preferably includes idiotypes of residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody fragments include an antigen-binding portion that can bind to a polypeptide having a sequence comprising residues 201 to 245 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody fragments include an antigen-binding portion that can bind to a polypeptide having a sequence comprising residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody fragments include an antigen-binding portion that can bind to a polypeptide having a sequence comprising residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).
Such antibody fragments can be generated by techniques known in the art. For example, such fragments include, but are not limited to, the F (ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of the F (ab')2 fragment, the Fab fragments that can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments. In a further technique, recombinant antibodies specific to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably, residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably, residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably, residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto, can be engineered and ectopically expressed in a wide variety of cell types. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO:1).

The antibodies used in the present methods can include "humanized" forms of non-human (e.g., murine) antibodies that are immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) which contain minimal amino acid residues derived from a non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human FR residues. Furthermore, a humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications can be made to further refine and optimize antibody performance.

The term "EphB4 protein" as used herein is taken to include full length EphB4 protein or a polypeptide fragment that comprises residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the EphB4 protein includes a polypeptide fragment that comprises residues 201 to 245 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the EphB4 protein includes a polypeptide fragment that comprises...
residues 220 to 244 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the EphB4 protein includes a polypeptide fragment that comprises residues 220 to 230 of EphB4 (SEQ ID NO: 1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

A EphB4 variant protein may be modified at the amino acid level and may include additions or deletions or replacements of amino acids which do not affect the functionality of the protein, such as conservative amino acid substitutions. An EphB4 protein may also include a truncated EphB4 protein. An EphB4 protein may be natural or recombinant. The EphB4 protein may be from any animal species, preferably the EphB4 protein is human.

An antibody or an antigen-binding portion thereof that is suitable for the methods of the present invention, preferably can inhibit cancerous growth of a cell by inhibiting the activity of an EphB4 protein. In the specification the term "cancerous growth" is taken to refer to abnormal and uncontrollable division and growth of a cell. Typically such a cell is identified as a cancer cell that may be able to invade and disrupt other tissues and has the potential to spread to other areas of the body. Cancerous growth of a cell can lead to the formation of a tumor that may be benign or malignant.

In the specification the term "cell(s)" is taken to include any cells. Preferably, the cells are derived from a mammalian species, such as, but not limited to, human, mice, bovine, sheep or domestic animals. It is preferred that the cells are selected from the group including, but not limited to, prostate cells, breast cells, colon cells, fibroblasts, epidermal cells, placental, liver, kidney, pancreas, heart, neural or muscle cells, or cancer or tumor cells. The cells may be normal cells, diseased cells, adult cells or embryonic cells. The cells may be single cells, cultured cells or part of a tissue. The cells may be genetically modified recombinant cells, such as a transgenic cell. Preferably, the cells express EphB4. The cells may be part of a whole animal. The cells may also be derived from a cell line. Preferably, the cells are from a cell line derived from, but not limited to, prostate, breast, colon or ovary cell line. The cell line is preferably selected from the group consisting of colon SW480, colon SW620, colon LIM1215, breast MCF7, breast T47-D, breast MDA-MB-231, breast MDA-MB-453, bladder J82, bladder T24, bladder RT119
and bladder 5637. More preferably, the cell line is selected from the group consisting of breast cancer cell line MCF-7 and colon cancer cell line SW480.

The antibody or an antigen-binding portion thereof of the present invention preferably can inhibit cancerous growth of one or more of cancer cells selected from the group consisting of breast cancer cells, prostate cancer cells, bowel cancer cells, bladder cancer cells, colon cancer cells, ovarian cancer cells, lung cancer cells, melanoma cells, lymphoma cells and leukemia cells.

The antibody or an antigen-binding portion thereof preferably specifically binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. In a preferred embodiment of the invention, at least one antibody or an antigen-binding portion thereof specifically binds to an epitope contained within residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto.

The term "specifically binds" in this specification, is to be understood to refer to binding characteristics of an antibody or an antigen-binding portion thereof which binds exclusively to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. The antibody or an antigen-binding portion thereof is preferably a polyclonal or monoclonal antibody that specifically binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody is an EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1).
The present invention provides a method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO:1).

Preferably, at least one antibody or an antigen-binding portion thereof binds to a polypeptide having a sequence comprising residues 201 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or an antigen-binding portion thereof binds to a EphB4 protein having a sequence comprising residues 201 to 245 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the antibody or an antigen-binding portion thereof binds to a EphB4 protein having a sequence comprising residues 220 to 244 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. More preferably, the antibody or an antigen-binding portion thereof binds to a EphB4 protein having a sequence comprising residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO:1).

The phrase "inhibiting cancerous growth of a cell" as used herein is taken to mean that cancerous growth of the cell is substantially reduced or prevented. In the present invention a cell is contacted with at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto, to result in the inhibition of cancerous growth of the cell as compared to an untreated cell. The method preferably results in the death of the cell. Preferably, the
sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

The present invention also provides a method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. In a preferred embodiment of the invention, at least one antibody or an antigen-binding portion thereof binds to an epitope contained within residues 201 to 245 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, at least one antibody or an antigen-binding portion thereof binds to an epitope contained within residues 220 to 244 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. More preferably, at least one antibody or an antigen-binding portion thereof binds to an epitope contained within residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

The phrase "inducing cell death of a cancer cell" is taken to mean that a cancer cell contacted with at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto, is caused to undergo cell death. Preferably, the antibody is an EphB4 polyclonal antibody (H-200 - Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1).

Cell death of a cancer cell may be assessed by a number of assays. For example, caspase-3 activation is considered to play a key role in the initiation of cellular events during cell death. Many different kits for the quantification of caspase-3 activity are available commercially. Mitochondrial membrane depolarization is often associated with the early stage of cell death. Changes in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition pores, which may play a central role in apoptosis. Depolarization can be detected by a number of different
assays including the use of Rhodamine 123, a green-fluorescent cationic dye that accumulates in active mitochondria, which have high membrane potentials allowing quick and easy detection of cellular disruption. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. LDH activity can easily be measured in culture supernatant by a single point assay using a spectrophotometric plate reader using commercially available kits. Elevated LDH in the culture medium is an indication of cell necrosis (death).

The morphology of a cell can also be examined to assess cell death. For instance, apoptosis is programmed cell death which is characterised by a series of typical morphological events, such as shrinkage of the cell and fragmentation into membrane-bound apoptotic bodies (17). These can be seen using a light microscope. In addition, a cell can be examined for the expression of genes related to cell death. In addition, RT-PCR analysis comparing EphB4 antibody treated and untreated cells from four different breast cancer cell lines has shown that EphB4 gene expression is down-regulated in treated cells.

A further aspect of the present invention is a method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1). The method preferably results in the death of a cancer cell in the subject.

The cancer is preferably selected from the group consisting of breast cancer, prostate cancer, bowel cancer, bladder cancer, colon cancer, ovarian cancer, lung cancer, melanoma, lymphoma and leukemia.

The subject treated by the methods of the invention may be selected from, but is not limited to, the group consisting of humans, sheep, cattle, horses, bovine, pigs, poultry, dogs and cats.
In the method an effective amount of at least one antibody or an antigen-binding portion thereof which binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto, is administered to a subject. In a preferred embodiment of the invention, at least one antibody or an antigen-binding portion thereof binds to an epitope contained within residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. The antibody or an antigen-binding portion thereof preferably specifically binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the antibody or an antigen-binding portion thereof is a polyclonal or monoclonal antibody.

The term "effective amount" means a dosage sufficient to provide treatment or prevention for the cancer being treated or prevented. This will vary depending on the subject and the type of cancer being effected. The effective amounts of at least one antibody or an antigen-binding portion thereof used in the methods of the present invention may vary depending upon the manner of administration, the condition of the animal to be treated, and ultimately will be decided by the attending scientist, Physician or veterinarian. The amount of antibody or an antigen-binding portion thereof used to treat or prevent a subject will also vary depending upon the nature and identity of the particular antibody or an antigen-binding portion thereof.

An antibody or an antigen-binding portion thereof is preferably administered to a subject by any suitable means known to those skilled in the art. Preferably, the antibody or an antigen-binding portion thereof can be contacted with a cell in numerous fashions, including, for example, intravenously.

Preferably, the antibody or an antigen-binding portion thereof of the present invention is combined with a suitable pharmaceutically-acceptable carrier or diluent to
form a pharmaceutical composition which may be suitable for administration to a human or animal subject. Suitable carriers or diluents include isotonic saline solutions, for example, phosphate-buffered saline. The pharmaceutical composition including at least one antibody or an antigen-binding portion thereof may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration. The antibody may be administered at a suitable dose dependent on the body weight of the subject. It is to be understood, however, that the routes of administration and dosages mentioned are intended to serve only as a guide since a person skilled in the art would be able to readily determine the optimum route of administration and dosage for any particular subject and cancer.

The antibody or an antigen-binding portion thereof used in the methods of the present invention may be combined with suitable excipients, such as emulsifiers, surfactants, stabilisers, dyes, penetration enhancers, anti-oxidants, water, salt solutions, alcohols, polyethylene glycols, gelatine, lactose, magnesium stearate and silicic acid. The antibody or an antigen-binding portion thereof is preferably formulated as a sterile aqueous solution. The antibody or an antigen-binding portion thereof can be combined with adjunct components that are compatible with the activity of the antibody. An antibody or an antigen-binding portion thereof used in the methods of the present invention may be preferably used to complement existing treatments for cancer. For example, the method of the present invention may also be used in combination with traditional cancer treatments such as radiotherapy, chemotherapy (eg using anthracyclines, 5FU, topoisomerase inhibitors, Cisplatin and Carboplatin), or hormone therapy or therapies utilising hormone modifiers (eg Catamoxifen).

In another aspect of the invention there is provided a method of identifying an agent which inhibits cancerous growth of a cell, the method comprising assessing the ability of the agent to bind to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. In a preferred embodiment of the invention, the agent binds to an epitope contained within residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1),
more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Preferably, the agent binds to a EphB4 protein having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto.

In the present specification the term "agent" is taken to include any molecule, compound or protein that can bind (interact with) residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Suitable agents can preferably include an antibody or an antigen-binding portion thereof that binds to a polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto. Most preferably, the agent is an antibody or an antigen-binding portion thereof that is a polyclonal or monoclonal antibody. The method preferably comprises assessing the ability of the agent to induce cell death of a cancer cell. The agent is preferably a EphB4 ligand, such as an antibody or an antigen-binding portion thereof, that is preferably specific for EphB4 protein and may be developed or obtained commercially for testing in in vitro or in vivo systems for its ability to inhibit cancerous growth of a cell.

For instance, antibodies or antigen-binding portions thereof directed to specific epitopes of residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at least 85%, preferably at least 90% identical thereto, can be tested for their ability to inhibit cancerous growth of a cell and preferably induce cell death. Dose response curves to assess the IC50 of the antibodies can be conducted to test efficacy of each antibody tested. In addition, antibody/receptor-ligand binding studies can be performed to assess the ability of the antibody to prevent ligand binding. Tyrosine phosphorylation of the EphB4 receptor following antibody binding can be assessed by
immunoprecipitation of the receptor with the respective antibody, followed by Western
analysis with an anti-phosphotyrosine antibody to confirm that the EphB4 receptor is
inactivated. The antibody with the best neutralising activity in terms of inhibiting
tyrosine phosphorylation and cell growth in vitro and preventing ligand binding to the
EphB4 receptor at the lowest 50% inhibitory concentration (IC₅₀) can be selected for
additional in vivo tests.

For instance, an in vivo model of metastasis and tumour growth using immu-
deficient NOD-SCID (non-obese diabetic, combined immunodeficiency) mice can be
used to test the ability of putative agents that can bind to a polypeptide having a
sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1), preferably residues
201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of EphB4 (SEQ ID
NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a sequence at
least 85%, preferably at least 90% identical thereto, for their efficacy as an anti-cancer
agent. Preferably, the sequence has a substitution of amino acid Asp (D) to Asn (N) at
residue 226 of EphB4 (SEQ ID NO:1). Moreover, a diverse array of tumor cell lines that
are available, most of which can be grown as xenografts, and these include the human
breast cancer cell line MCF-7 and colon cancer cell line HT29, can be used for in vitro
testing. Xenograft tumours can be grown in the mouse model either after subcutaneous
injection, where they will grow as a mass, or after injection into the tail vein allowing
mimicry of the hematogenous spread of metastasis that results in secondary deposits in
other organs. Once suitable engraftment periods and inoculation doses for each cell line
have been established, the model can be used to test various agents that bind to a
polypeptide having a sequence comprising residues 200 to 400 of EphB4 (SEQ ID NO:1),
preferably residues 201 to 245 of EphB4 (SEQ ID NO:1), preferably residues 220 to 244 of
EphB4 (SEQ ID NO:1), more preferably residues 220 to 230 of EphB4 (SEQ ID NO:1), or a
sequence at least 85%, preferably at least 90% identical thereto. Cells may also be treated
with sub-lethal doses of a chosen EphB4 antibody equating to the IC₅₀ and IC₇₅ to assess
the engraftment of treated cells compared with non-treated cells. This will assess the
effects of reduced functional expression of EphB4 on establishment and metastasis of
tumour cell lines.

The in vivo models can also be used for pre-clinical assessment of potential new
therapies for treatment of EphB4 positive tumours cell lines. The use of subcutaneous
injection will allow the examination of tumours that have been allowed to establish for different periods of time. This can be used to determine the ability of an agent, such as an antibody or an antigen-binding portion thereof, to ablate newly and well-established tumours compared to vehicle control. The use of tail vein injections can be used to determine whether treatment with an antibody or an antigen-binding portion thereof will reduce number of metastases formed as a result of hematogenous spread. The agents identified by the methods of the present invention may be used for treatment or prevention of cancer. The present invention also provides an agent identified by the method described above.

Throughout this specification the word "comprise", or variations such as "comprised" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The invention will hereinafter be described by way of the following non-limiting Figures and Examples.

**Example 1 – Immunohistochemical localisation of EphB4:**

An EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) was used to analyse the localisation of the EphB4 protein in tumour and normal tissue. Colon and breast tissue showed marked increase in the levels of this protein in the tumour epithelial cells when compared with the matched normal tissue (as shown in Figure 1). The demonstration of high expression of *EphB4* on the tumour epithelial cells in two of the most commonly occurring cancers suggests that EphB4 is critical to the progression of these tumours.

**Example 2 – RT-PCR expression of EphB4:**

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to compare expression of *EphB4* (1187 bp) and internal 18S rRNA (489 bp) in five tumour (T) / normal (N) pairs (results shown in Figure 2). Analysis of 63 colon cancers from 60 patients indicated that *EphB4* is over-expressed in the tumour tissue of 80% of patients, implying broad application as a therapeutic target (Figure 2). The differential expression
between tumour cells and normal tissue suggests anti-EphB4 tumour therapy may have a preferential effect on colon (and other) tumours.

A comparison of the expression profile of EphB4 with that of other receptor protein tyrosine kinases already being targeted in clinical trials (HER2, EGFR and VEGFR) suggests that EphB4 is expressed to a lesser degree in normal tissues. Information from EST databases suggests that low level expression of EphB4 may be present in kidney, ovary and placenta, and very low level expression in heart, lung, peripheral nerves and vascular tissue. Accordingly therapies that target EphB4 may be expected to produce less side effects than those that target other receptor tyrosine kinases.

**Example 3 - EphB4-specific antibody studies:**

A direct tumoricidal effect of an EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) *in vitro* was demonstrated. Incubation of a fully confluent monolayer of colon cancer cell lines SW480 and SW620 with a 1/500 dilution of EphB4 antibody H-200 (Santa Cruz Biotechnology - Lot numbers B141 and F182) caused the cells to lift of the bottom of the culture vessel and die (Figure 3). This was a common response seen when treating colon cancer cell lines including SW480, SW620, LIM1215, breast cancer cell lines MCF-7, T47-D, MDA-MB-453, MDA-MB-231 and Hs578t, bladder cancer cell lines J82, RT119, 5637 and T24.

Incubation of the breast cancer cell line MCF-7 and the colon cancer cell line SW480 with three different concentrations of antibody (2 μg/ml, 1 μg/ml and 0.2 μg/ml) resulted in cell death in a dose dependent manner (see Figure 4 and Figure 5). This effect was not seen following exposure of the endothelial cell line HUVEC-C to the EphB4 antibody. Analysis of the caspase-3 activity suggested that cell death was not *via* apoptosis (Figure 6). These results suggest that by cross-linking or binding to membrane receptors, antibodies may mimic or modulate receptor activity and that these antibodies-generated transmembrane signals might cause apoptosis or growth inhibition (18). Possible alternative mechanisms for the induction of cell death include ras-mediated non-apoptotic cell death or restoration of gap junction intercellular communication.
(GJIC), a direct cell-cell communication pathway that is known to be prevented by Eph receptor signaling (19).

Polyclonal antibodies specific for EphB4 have been developed and are available commercially, for testing of these antibodies in *in vitro* and *in vivo* systems. Figure 7 shows results of the percentage viability of breast cancer cells after treatment with five EphB4 antibodies as detailed below:

1. a EphB4 polyclonal antibody (Swiss) directed to amino acid residues 825 to 991 of the carboxy terminus of mouse EphB4;

2. a polyclonal N-terminal EphB4 antibody (N-19 Santa Cruz Biotechnology) directed to the N-terminal first 19 amino acids of the EphB4 amino acid sequence which is likely to be amino acids residues 16 to 34 of the mature EphB4 (SEQ ID NO:1);

3. a polyclonal EphB4 C-terminal antibody (C-16 Santa Cruz Biotechnology) directed to the carboxy-terminal corresponding to tyrosine kinase domain consisting of amino acid residues 615 to 874 of EphB4 (SEQ ID NO:1);

4. a EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1);

5. a EphB4 polyclonal antibody (H-200 (old) -Santa Cruz Biotechnology -Lot number B141 batch) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1).

Cell death effect was seen in treatment with the H-200 antibody specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). A comparison of cells grown in medium with or without active complement proteins shows that complement plays no role in the Ab-mediated cell death (see Figure 7).

The mechanism of cell death can be further by analysing changes to gene expression induced in cancer cells *in vitro* after incubation with sub-lethal doses of the H-200 EphB4 antibody using microarray techniques.
Example 4 – Expression of EphB4 in human tissue:

Information from EST databases and Northern analysis of normal human tissues (20) suggests that low level expression of EphB4 may be present in kidney, ovary and placenta, and very low level expression in heart, lung, peripheral nerves and vascular tissue, and no expression in brain. In order to determine whether gene expression correlates with the level of protein actually translated a western analysis using these tissues was performed with an EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). By comparison with a single colon tumour sample it was shown that the level of gene expression does not correspond to the amount of EphB4 protein produced in these tissues (Figure 8). The differential expression between tumour cells and normal tissue suggests that anti-EphB4 tumour therapy may have a preferential effect on colon tumours.

Several hybridising bands were obtained with Western blot analysis (data not shown). However, the identity of these protein bands that are specific to the tumour sample(s), and common to all tumour samples tested, remains to be determined but may correspond to splice variation. Two EphB4 splice variants (named EphB4v1 and EphB4v2) were identified. Determination of the EphB4 gene structure has shown that EphB4v1 results from the absence of the 53 amino acids encoded by the entire exon 16 (Figure 9). If EphB4v1 is translated the resultant protein would lack a portion of both the kinase and SAM domains, two protein domains that are known to have roles in transmitting signals to intracellular targets. EphB4v2 is caused by the in-frame deletion of the entire exon 6 which results in the absence of 111 amino acids. EphB4v2 would encode a protein that lacks the first fibronectin type III repeat. The role of fibronectin type III repeats is not known and the effect of removing one of these repeats is unknown at this time.

Example 5 – RT-PCR analysis of EphB4:

Because of EphB4 possible role in angiogenesis, the expression of EphB4 in endothelial cells using RT-PCR was performed. Low level of expression of EphB4 was observed. However, when these cells were grown in the presence of the EphB4
polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) there was no apparent change in cell growth or morphology, even at the highest concentration of antibody. The effect of the EphB4 antibody on the growth and morphology of NIH3T3 cells was also tested. There was no morphological or growth response (data not shown). The nontumorigenic breast cell line MCF10A, established from mammary tissue from a 36-year old Caucasian patient with fibrocystic breast disease (21) was also tested and was observed to express EphB4 at low levels but did not respond to the anti-EphB4 antibody (data not shown). These results, when considered together with results gained from the western analysis of normal human tissues (Example 5), suggest that tissues that show a high level of EphB4 expression will be negatively affected by an EphB4 antibody.

The results indicated that after treatment with the EphB4 polyclonal antibody (H-200 -Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1) the EphB4 gene is down-regulated (Figure 10). This agrees with the finding that most antibodies increase RTK down-regulation and internalisation of the receptor after blocking the ligand-receptor interaction and inhibiting ligand-induced RTK signaling. This is since certain antibodies can influence tumour growth by altering the intracellular signaling pattern inside the targeted tumour cell. The analysis of global changes in gene expression in Ab-treated cells compared to untreated cells may identify genes involved in EphB4-related signaling.
Example 6 – EphB4 antibody- Epitope mapping:

A EphB4 commercially available H-200 polyclonal Ab from Santa Cruz is raised against a recombinant protein corresponding to amino acids 201-400 of SEQ ID NO: 1 of EphB4 human receptor. The sequence includes the cysteine-rich domain and the first fibronectin type III repeat and accordingly it was expected that several different antigenic regions would be recognised. Six blocking peptides of 25 amino acids (overlap by 5 amino acids, offset by 20 amino acids) were designed against specific amino acid residues of EphB4 protein (SEQ ID NO:1) as indicated in Figure 11. The blocking peptides have the following amino acid sequence:

Peptide 1  SEQ ID NO:2: TVNLTRFPETVPRELVPVAGSCVV
Peptide 2  SEQ ID NO:3: GSCVVDAVPAGPSPLYCREDGQW
Peptide 3  SEQ ID NO:4: EDGQWAEPVTGCSCAPFFAEGN
Peptide 4  SEQ ID NO:5: AEAGNTKCRACQAQTFKPLSGEGSC
Peptide 5  SEQ ID NO:6: GEGSCQPCPANSHTIGSAVCQCR
Peptide 6  SEQ ID NO:7: VCQCRVGYFRATDPRGAPCTTPPS

The peptides were tested for their ability to prevent cell death after pre-incubation with the EphB4 polyclonal antibody (H-200 - Santa Cruz Biotechnology) specifically directed to the extracellular domain amino residues 201 to 400 of EphB4 (SEQ ID NO:1). A cocktail of all the peptides was initially tested and successfully prevented cell death as determined using trypan blue exclusion (Figure 12) and caspase-3 activity assays (Figure 13). The peptides were then tested separately and partial rescue was observed for Peptide 1 (SEQ ID NO:2) and Peptide 2 (SEQ ID NO:3) (see Figure 14).

Peptide 1 and Peptide 2 overlap by five amino acids residues (GSCVV designated SEQ ID NO:13) indicating that this amino acid sequence corresponding to residues 221 to 225 of EphB4 (SEQ ID NO:1) is possibly the core of the reactive epitope.

Because the initial experiment with the cocktail of peptides effectively contained twice the molar amount of the GSCVV (SEQ ID NO:13) sequence (ie residues 221 to 225 of EphB4 (SEQ ID NO:1)) a further blocking experiment in which twice the amount of each of Peptide 1 (SEQ ID NO:2) and Peptide 2 (SEQ ID NO:3) was compared with the
initial amount of the peptides together. All treatments were successful in preventing tumour cell death caused by the EphB4 polyclonal antibody (H-200 from Santa Cruz) (see Figure 15).

Three new peptide (Peptides 7 to 9) of different lengths that span the GSCVV (SEQ ID NO:13) core epitope sequence were made commercially based on specific amino acid residues of EphB4 protein (SEQ ID NO:1) as indicated in Figure 16. The blocking peptides have the following amino acid sequence:

Peptide 7  SEQ ID NO:8:  AGSCVVDA  
Peptide 8  SEQ ID NO:9:  VAGSCVVDAV  
Peptide 9  SEQ ID NO:10:  LVVPVAGSCVVDAVPA  

However, due to the high number of hydrophobic amino acids in peptides 8 and 9, these peptides were not soluble in any solution that could be applied to cells, thus preventing further testing. However, Peptide 7 (SEQ ID NO. 8) with an amino acid sequence corresponding to residues 220 to 227 of EphB4 (SEQ ID NO:1) was used in blocking experiments and was able to rescue cells from the cell death effect caused by the addition of the EphB4-antibody (H-200 from Santa Cruz) alone (see Figure 17).

All publications discussed above are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or any other country before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.
References:


CLAIMS

1. A method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1).

2. A method according to claim 1, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1).

3. A method according to claim 1 or 2, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 244 of EphB4 (SEQ ID NO: 1).

4. A method according to any one of claims 1 to 3, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1).

5. A method for inhibiting cancerous growth of a cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located in a sequence at least 85% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

6. A method according to claim 5, wherein the antibody or antigen-binding portion thereof binds to an epitope located in a sequence at least 90% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

7. A method according to any one of claims 1 to 6, wherein the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

8. A method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof,
wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1).

9. A method according to claim 8, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1).

10. A method according to claim 8 or 9, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 244 of EphB4 (SEQ ID NO: 1).

11. A method according to any one of claims 8 to 10, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1).

12. A method for inducing cell death of a cancer cell, the method comprising contacting the cell with at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located in a sequence at least 85% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

13. A method according to claim 12, wherein the antibody or antigen-binding portion thereof binds to an epitope located in a sequence at least 90% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

14. A method according to any one of claims 8 to 13, wherein the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

15. A method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 200 to 400 of EphB4 (SEQ ID NO: 1).
16. A method according to claim 15, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 201 to 245 of EphB4 (SEQ ID NO: 1).

17. A method according to claim 15 or 16, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 244 of EphB4 (SEQ ID NO: 1).

18. A method according to any one of claims 15 to 17, wherein the antibody or antigen-binding portion thereof binds to an epitope located within residues 220 to 230 of EphB4 (SEQ ID NO: 1).

19. A method for treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of at least one antibody or an antigen-binding portion thereof, wherein the antibody or antigen-binding portion thereof binds to an epitope located in a sequence at least 85% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

20. A method according to claim 19, wherein the antibody or antigen-binding portion thereof binds to an epitope located in a sequence at least 90% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

21. A method according to any one of claims 15 to 20, wherein the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

22. A method of identifying an agent which inhibits cancerous growth of a cell, the method comprising assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 200 to 400 of EphB4 (SEQ ID NO:1).

23. A method according to claim 22, wherein the method comprising assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 201 to 245 of EphB4 (SEQ ID NO:1).
24. A method according to claim 22 or 23, wherein the method comprising assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 220 to 244 of EphB4 (SEQ ID NO: 1).

25. A method according to any one of claims 22 to 24, wherein the method comprising assessing the ability of the agent to bind to an EphB4 polypeptide within the region of residues 220 to 230 of EphB4 (SEQ ID NO: 1).

26. A method of identifying an agent which inhibits cancerous growth of a cell, the method comprising assessing the ability of the agent to bind to a polypeptide comprising a sequence at least 85% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

27. A method according to claim 26, the method comprising assessing the ability of the agent to bind to a polypeptide comprising a sequence at least 90% identical to residues selected from the group consisting of residues 200 to 400 of EphB4 (SEQ ID NO: 1), residues 201 to 245 of EphB4 (SEQ ID NO: 1), residues 220 to 244 of EphB4 (SEQ ID NO: 1) and residues 220 to 230 of EphB4 (SEQ ID NO: 1).

28. A method according to any one of claims 22 to 27, wherein the sequence has a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

29. An agent which inhibits cancerous growth of a cell when identified by the method according to any one of claims 22 to 28.

30. A purified EphB4 antibody which binds to a polypeptide having a sequence at least 85% identical to residues 201 to 245 of EphB4 (SEQ ID NO: 1).

31. A purified EphB4 antibody according to claim 30, wherein the polypeptide has a sequence at least 90% identical to residues 201 to 245 of EphB4 (SEQ ID NO: 1).

32. A purified EphB4 antibody which binds to a polypeptide having a sequence at least 85% identical to residues 220 to 244 of EphB4 (SEQ ID NO: 1).

33. A purified EphB4 antibody according to claim 32, wherein the polypeptide has a sequence at least 90% identical to residues 220 to 244 of EphB4 (SEQ ID NO: 1).
34. A purified EphB4 antibody which binds to a polypeptide having a sequence at least 85% identical to residues 220 to 230 of EphB4 (SEQ ID NO: 1).

35. A purified EphB4 antibody according to claim 34, wherein the polypeptide has a sequence at least 90% identical to residues 220 to 230 of EphB4 (SEQ ID NO: 1).

36. A purified EphB4 antibody according to any one of claims 30 to 35, wherein the antibody binds to an epitope located in residues 200 to 400 of EphB4 (SEQ ID NO: 1).

37. A purified EphB4 antibody according to any one of claims 30 to 36, wherein the antibody binds to a polypeptide having a substitution of amino acid Asp (D) to Asn (N) at residue 226 of EphB4 (SEQ ID NO: 1).

38. A purified EphB4 antibody according to any one of claims 30 to 37, wherein the antibody is a monoclonal antibody.
Figure 1

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<th>Poorly Differentiated</th>
<th>Moderately-well Differentiated</th>
<th>Well Differentiated</th>
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<tr>
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<td>C. Tumour</td>
<td>E. Tumour</td>
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<tr>
<td>B. Matched Normal Mucosa</td>
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<td>F. Matched Normal Mucosa</td>
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</table>
Figure 2

[Image of a gel electrophoresis diagram showing bands at 1187 bp and 489 bp]
Figure 3

<table>
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<tr>
<th>Control</th>
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<th>EphB4 antibody</th>
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<td>H-200 (Santa Cruz</td>
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<tr>
<td>Biotechnology)</td>
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</tr>
<tr>
<td>Lot # B141</td>
<td>Lot # F182</td>
<td></td>
</tr>
</tbody>
</table>

SW620

SW480
Figure 4

Cell count after 48 h

Concentration of Antibody Used

0 10 20 30 40 50 60 0 10 20 30 40 50 60

no Ant 0.2 mg/ml 1 mg/ml 2 mg/ml
Figure 5

Dilution of Antibody used
Figure 6

- Graph showing Caspase-3 activity per cell (x 100000) against Treatment (Control, 0.2 μg/ml, 1 μg/ml, 2 μg/ml) for time points 24, 48, and 72 hours.
Figure 7

Percentage Viability of Breast Cancer Cells After 65 h Treatment with EphB4 Antibodies

EphB4 Antibody Used

CLM Experiment
FCS Experiment
Figure 8

A

Marker Brain Heart Kidney Liver Lung Breast Placenta Colon Tumour

B

220 kDa
130 kDa
90 kDa
70 kDa

Marker Brain Heart Kidney Liver Lung Breast Placenta Colon Tumour
Figure 9

Wild-type EphB4 receptor

Globular Domain
Cysteine Rich Region
Fibronectin Type III repeats
Transmembrane domain
Tyrosine Kinase domain
SAM domain
PDZ domain

EphB4 v1 receptor

EphB4 v2 receptor

*
Figure 10

Marker 1  Marker 2  231 Control  231 Treated  468 Control  468 Treated  Hs 5781 Control  Hs 5781 Treated  MCF7 Control  MCF7 Treated  T47D Control  T47D Treated  RT-ve

EphB4

PBGD
Figure 11

201 TVNLTRFPETVPRELVVPVAGSCVVDAPAPGSPSLYCREDGQWAEPVPVTGCS  254
TVNLTRFPETVPRELVVPVAGSCVV  (SEQ ID NO:2- Peptide 1)
GSCVVDAPAPGSPSLYCREDGQW  (SEQ ID NO:3- Peptide 2)
       EDGQWAEPVPVTGCS-

255 CAPGFEAAEGNTKCRACAGTFKPLSLEGSCQPCPANSNSNTIGSAVCQCRVGY  308
CAPGFEAAEGN  (SEQ ID NO:4- Peptide 3)
       AAEGNTKCRACAGTFKPLSLEGSC  (SEQ ID NO:5- Peptide 4)
       GEGSCQPCPANSNSNTIGSAVCQCR  (SEQ ID NO: 6-
Peptide 5)
       VCQCRVGY-

309 FRARTDPRGAPCTTPSAPRVSRLNGSSLHLEWSAPLEGRETDLTYALRCR  362
FRARTDPRGAPCTTPS  (SEQ ID NO:7- Peptide 6)
Figure 12

![Graph showing percentage viability for different treatments](image)

**Treatment**

- Untreated 1
- Untreated 2
- Ab only 1
- Ab only 2
- Ab + all peptides 1
- Ab + all peptides 2
Figure 13

![Graph showing relative level of Caspase-3 activity per viable cell for different treatments. The treatments include Untreated 1, Untreated 2, Ab only 1, Ab only 2, Ab + all peptides 1, and Ab + all peptides 2. The graph indicates a comparison of the activity levels across these treatments.](image-url)
Figure 14

[Bar chart showing percentage viability of different treatments. The x-axis represents different treatments, including Control, and various combinations of Ab and Peptide labeled as 1, 2, 3, 4, 5, 6, and Ab alone.]
Figure 15

![Graph showing percentage viability for different treatments.](image)

- **Control**
- **Ab + 10 μl Peptide 1**
- **Ab + 10 μl Peptide 2**
- **Ab + 5 μl Peptide 1 + 5 μl Peptide 2**
- **Ab Alone**

**Treatment**

**Percentage Viability**

- **Marker 1**
- **Marker 2**
Figure 16

201  TVNLTRFPETVPRELVVPAGSCVVDAPAPGSPSLYCREDGQWAEQPVTGCS 254
TVNLTRFPETVPRELVVPAGSCVV (SEQ ID NO:2- Peptide 1)

GSCVVDAVPAPGSPSLYCREDGQW (SEQ ID NO:3- Peptide 2)

AGSCVVDA  (SEQ ID NO:8- Peptide 7)
VAGSCVVDAV  (SEQ ID NO:9-Peptide 8)
LVVPVAGSCVVDAVP  (SEQ ID NO:10- Peptide 9)
Figure 17

Peptide 7 can abrogate the effects of the EphB4-specific antibody
### Figure 18

**SEQ ID NO:1**

Homo Sapiens Ephrin type-B receptor 4 Precursor (EphB4)

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**SUBSTITUTE SHEET (RULE 26)**
Figure 19

N-19
- Globular Domain
- Cysteine Rich Region

H-200
- Fibronectin Type III repeats
- Transmembrane domain

C-16
- Tyrosine Kinase domain
- SAM domain
- PDZ domain
Figure 20

TVNLTRFPETVPRELVPVAGSCVVDAVPAPGPSPSLYCREDGQWAEQPVTGCS 254
AGSCVVDAVPAPGPSPSLYCREDGQ (Peptide 11, SEQ ID NO:12)

AGSCVVNAVAPGPSPSLYCREDGQ (Peptide 10, SEQ ID NO:11)

Aspartate (D)  Asparagine (N)