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(54) Titre : BIOMARQUEUR SPECIFIQUE DU MELANOME
(54) Title: MELANOMA SPECIFIC BIOMARKER

Fig 1

Melanoma | Normal tissue | Normal adjacent tissue

(57) Abrégé/Abstract:
Described are melanoma specific biomarkers comprising the nucleic acid sequence of the Engrailed-2 (EN2) gene or the amino acid sequence of the encoded EN2 protein. Also described are uses of the biomarkers in the treatment, diagnosis, monitoring and imaging of melanoma.

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(54) Title: MELANOMA SPECIFIC BIOMARKER

Fig 1

(57) Abstract: Described are melanoma specific biomarkers comprising the nucleic acid sequence of the Engrailed-2 (EN2) gene or the amino acid sequence of the encoded EN2 protein. Also described are uses of the biomarkers in the treatment, diagnosis, monitoring and imaging of melanoma.

WO 2011/067549 A1

MELANOMA SPECIFIC BIOMARKER

The present application relates to biomarkers, in particular to biomarkers for melanoma.

Melanoma is a type of cancer of the skin that begins in melanocytes. It may begin in a mole but can also begin in other pigmented tissues such as in the eye or in the intestines. Malignant melanoma is relatively rare, accounting for 10% of all skin cancer cases. However, malignant melanoma is also responsible for the most deaths. In England and Wales, approximately 1,500 people die every year due to malignant melanoma.

Despite advances in technology, melanoma remains difficult to treat, especially if not caught at an early stage.

SUMMARY OF THE INVENTION

According to one aspect of the present invention, there is provided a melanoma specific biomarker, the biomarker comprising:-

(i) a nucleic acid sequence comprising SEQ ID NO:1, or a fragment or variant thereof, or a nucleic acid molecule which comprises said nucleic acid sequence; or

(ii) an amino acid sequence comprising SEQ ID NO:2, or a fragment or variant thereof, or an amino acid molecule which comprises said amino acid sequence.

In this respect, SEQ ID NO:1 corresponds to the nucleic acid sequence of the Engrailed-2 (EN2) gene (GenBank reference number NM_001427) and SEQ ID NO:2 corresponds to the EN2 protein encoded thereby (NCBI accession number P19622, gi21903415).

Surprisingly, it has been found that the EN2 gene is significantly up-regulated in melanoma.

The EN2 gene encodes a homeodomain-containing transcription factor that has a number of important functions in early development including axonal guidance and boundary

formation (reviewed in Morgan R, (2006). Engrailed: Complexity and economy of a multi-functional transcription factor. FEBS letters 580, 2531-2533, which is incorporated herein by reference in its entirety). Its NCBI/GenBank reference number is NM_001427. It has previously been reported to act as an oncogene in breast cancer, although no diagnostic significance has been attributed to it (Martin, N.L., Saba-El-Leil, M.K., Sadekova, S., Meloche, S. and Sauvageau, G. (2005) EN-2 is a candidate oncogene in human breast cancer. Oncogene 24, 6890-6901, which is incorporated herein by reference in its entirety). The EN2 gene product is a 33kDa protein (EN2).

Preferably, the fragments or variants thereof comprise:-

(i) a nucleic acid sequence that has at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% nucleic acid sequence identity with SEQ ID NO:1, a nucleic acid sequence that is hybridizable thereto under stringent conditions, and/or a nucleic acid sequence that is complementary thereto;

(ii) an amino acid sequence that has at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% amino acid sequence identity with SEQ ID NO:2, or

(iii) an amino acid sequence encoded by a nucleic acid sequence of (i).

Put another way, in accordance with part (iii) above, it is preferred that the fragments or variants thereof comprise:-

(A) an amino acid sequence encoded by a nucleic acid sequence, wherein said nucleic acid sequence has at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% nucleic acid sequence identity with SEQ ID NO:1;

(B) an amino acid sequence encoded by a nucleic acid sequence, wherein said nucleic acid sequence is hybridizable under stringent conditions to a nucleic acid sequence that has at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least

about 96%, or at least about 97%, or at least about 98%, or at least about 99% nucleic acid sequence identity with SEQ ID NO:1; or

(C) an amino acid sequence encoded by a nucleic acid sequence, wherein said nucleic acid sequence is complementary to a nucleic acid sequence that has at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% nucleic acid sequence identity with SEQ ID NO:1.

Preferably, the fragments thereof comprise (i) at least four, preferably at least five, preferably at least six, preferably at least seven, preferably at least eight consecutive amino acids from SEQ ID NO:2 or (ii) a fragment of the nucleic acid sequence of SEQ ID NO:1 which encodes at least four, preferably at least five, preferably at least six, preferably at least seven, preferably at least eight consecutive amino acids from SEQ ID NO:2. Longer fragments are also preferred, for example at least about 10, 15, 20, 25, 30, 50, 75, 100, 150, 200, 225 and up to at least about 250 amino acids of SEQ ID NO:2 or corresponding coding fragments of SEQ ID NO:1. Fragments may also include truncated peptides that have x amino acids deleted from the N-terminus and/or C-terminus. In such truncations, x may be 1 or more (i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more), but preferably less than 150 amino acids of SEQ ID NO:2 or corresponding coding fragments of SEQ ID NO:1.

Preferably, the fragments or variants thereof are functional fragments or variants thereof.

According to another aspect of the present invention, there is provided a method for diagnosing melanoma in a patient or for identifying a patient at risk of developing melanoma, the method comprising:

(a) determining an amount of the melanoma specific biomarker in a sample obtained from a patient;

(b) comparing the amount of the determined melanoma specific biomarker in the sample from the patient to the amount of the melanoma specific biomarker in a normal control;

wherein a difference in the amount of the melanoma specific biomarker in the sample from the patient compared to the amount of the melanoma specific biomarker in the normal control is associated with the presence of melanoma or is associated with a risk of developing melanoma.

According to another aspect of the present invention, there is provided a method for monitoring the progression of melanoma in a patient, the method comprising:

(a) determining an amount of the melanoma specific biomarker in a sample obtained from a patient;

(b) comparing the amount of the determined melanoma specific biomarker in the sample from the patient to the amount of the melanoma specific biomarker in a normal control; and

(c) repeating steps (a) and (b) at two or more time intervals,

wherein an increase in the amount of the melanoma specific biomarker from the patient over time is associated with an increase in the progression of melanoma and a decrease in the amount of the melanoma specific biomarker from the patient over time is associated with a decrease in the progression of melanoma.

Accordingly, the methods of the present invention can be used to detect the onset, progression, stabilisation, amelioration and/or remission of melanoma.

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

Accordingly, in one example of the present invention, there is provided a method of diagnosing or monitoring the progression of melanoma, comprising detecting and/or quantifying the melanoma specific biomarker in a biological fluid obtained from a patient.

As discussed above, it is preferred that at least two detection and/or quantification steps are provided, spaced apart temporally.

Preferably, the steps are spaced apart by a few days, weeks, years or months, to determine whether the levels of the melanoma specific biomarker have changed, thus indicating whether there has been a change in the progression of the cancer, enabling comparisons to be made between a level of the biomarker in samples taken on two or more occasions, as an increase in the level of the biomarker over time is indicative of the onset or progression of the cancer, whereas a decrease in the level of the biomarker may indicate amelioration and/or remission of the cancer.

Preferably, the difference in the level of the biomarker is statistically significant, determined by using a "t-test" providing confidence intervals of preferably at least about 80%, preferably at least about 85%, preferably at least about 90%, preferably at least about 95%, preferably at least about 99%, preferably at least about 99.5%, preferably at least about 99.95%, preferably at least about 99.99%.

The biomarkers and methods of the invention are particularly useful in detecting early stage cancer and are more sensitive than known methods for detecting early stage melanoma. Thus, the biomarkers and methods of the invention are particularly useful for confirming cancer when a patient has tested negative for cancer using conventional methods.

Prognosis and choice of treatment are dependent upon the stage of the cancer and the patient's general state of health.

Stage 1 of melanoma is thin and the epidermis usually appears scraped. This stage of skin cancer is subdivided into two other categories. These additional categories describe the thickness of the tumour. Stage 1a is less than 1.0 mm and has no ulceration. Stage 1b is less than 1.0 mm but has ulceration. It is also considered to be in stage 1b if it is 1.01 - 2.0 mm even if it does not involve ulceration. In this stage and stage 2 the melanoma has not yet spread to the lymph nodes.

Stage 2 is also subdivided into three more categories that signify the thickness and the existence or non-existence of ulceration. The tumour in stage 2a is 1.01 - 2.0 mm with

ulceration or 2.01 - 4.0 mm without ulceration. Stage 3b has a tumour thickness of 2.01mm with ulceration or a thickness of more than 4.0mm without ulceration.

When this type of skin cancer advances to stage 3 a significant change occurs. At this stage, the melanoma tumour has spread to the lymph nodes. This is a much more serious stage of the disease because when healthy, the lymph nodes fight disease, cancer and some other infections.

Patients with stage 3 of this cancer have melanoma that has spread into lymph nodes near the primary tumour. This stage also involves in-transit metastasis that has skin or connective tissue that is more than 2 cm from the original tumour. However, at this point it has not spread past the regional lymph nodes.

In stage 4, the melanoma has spread to lymph nodes that are a distance from the original tumour or to internal organs. These organs are most often the lung, liver, brain, bone and then the gastrointestinal tract.

It will be appreciated that the term "early stage" as used herein can be said to refer to stage 1 and/or stage 2 of melanoma, as discussed above.

With regard to the term "late stage" as used herein, it will be appreciated that this term can be said to refer to stage 3 and/or stage 4 of melanoma.

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

In one aspect, there are provided methods according to the present invention for detecting early stage cancer, wherein an increase between the control and the sample obtained from the patient is indicative of early stage cancer. Preferably, the increase is at least about 50%, preferably at least about 60%, preferably at least about 70%, preferably at least about 80%, preferably at least about 90%, preferably at least about 100%, preferably at least about

200%, preferably at least about 300%, preferably at least about 400%, preferably at least about 500%, preferably at least about 1000%.

Also provided are methods according to the present invention for detecting late stage cancer wherein an increase between the control and the sample obtained from the patient is indicative of late stage cancer. Preferably, the increase is at least about 75%, preferably at least about 85%, preferably at least about 100%, preferably at least about 110%, preferably at least about 125%, preferably at least about 150%, preferably at least about 200%, preferably at least about 300%, preferably at least about 400%, preferably at least about 500%, preferably at least about 1000%..

Further provided are methods according to the present invention for monitoring a change in stage of cancer, wherein an increase, relative to an earlier stage sample or control is indicative of progression of the cancer from an earlier stage to later stage of disease, for example from from stage 1 to stage 2, from stage 2 to stage 3, from stage 3 to stage 4, or from early stage to late stage. The increase may also be indicative of progression of the cancer between subcategories of each stage, for example from stage 1a to stage 1b, or from stage 2a to stage 2b. Preferably, the increase is at least about 50%, preferably at least about 60%, preferably at least about 70%, preferably at least about 80%, preferably at least about 90%, preferably at least about 100%.

It is preferred that the melanoma specific biomarker is indicative of the presence of melanoma or the risk of developing melanoma when present at a level of at least about 1.5-fold, preferably at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, preferably at least about 20-fold, preferably at least about 30-fold, preferably at least about 40-fold, preferably at least about 50-fold, preferably at least about 100-fold, preferably at least about 150-fold, preferably at least about 200-fold that of a normal control.

Also provided by the present invention is a method for monitoring the efficacy of a treatment for melanoma, comprising detecting and/or quantifying the presence of the melanoma specific biomarker in a biological sample obtained from a patient.

Preferably, in the methods of the present invention, detection and/or quantification of the melanoma specific biomarker is by one or more of MALDI-TOF, SELDI, via interaction with a ligand or ligands, 1-D or 2-D gel-based analysis systems, Liquid Chromatography, combined liquid chromatography and Mass spectrometry techniques including ICAT(R) or iTRAQ(R), thin-layer chromatography, NMR spectroscopy, sandwich immunoassays, enzyme linked immunosorbent assays (ELISAs), radioimmunoassays (RAI), enzyme immunoassays (EIA), lateral flow/immunochromatographic strip tests, Western Blotting, immunoprecipitation, particle-based immunoassays including using gold, silver, or latex particles, magnetic particles or Q-dots, and immunohistochemistry on tissue sections.

Preferably, detection and/or quantification of the melanoma specific biomarker is performed on a microtitre plate, strip format, array or on a chip.

Preferably, detection and/or quantification of the melanoma specific biomarker is by an ELISA comprising antibodies specific for the melanoma specific biomarker, preferably linked to a reporter.

Preferably, detection and/or quantification of the melanoma specific biomarker is by a biosensor.

Preferably, the sample comprises biological fluid or tissue obtained from the patient. Preferably, the biological fluid comprises blood, serum, plasma or lymph. Preferably, the tissue comprises cells obtained from the tumour itself or surrounding cells. For example, the tissue may comprise cells from a lesion or mole. Preferably, the tissue comprises cells which have been scraped from the surface of a lesion or mole.

In one example of the methods described herein, detection and/or quantification of the melanoma specific marker comprises scraping cells from a lesion or mole and incubating said cells with an anti-EN2 antibody linked to a detectable marker. Preferably, the cells are then washed in a wash solution to remove unbound antibody. The results obtained with the cells scraped from a lesion or mole may then be compared with a normal control.

It is also preferred that the biological fluid is substantially or completely free of whole/intact cells. Preferably the biological fluid is free of platelets and cell debris (such as that produced upon the lysis of cells). Preferably the biological fluid is free of both prokaryotic and eukaryotic cells.

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

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

Another aspect of the present invention relates to a method for treating a patient with melanoma, the method comprising administering to a patient a *therapeutically effective* amount of (i) a biomarker of the present invention or (ii) an antibody or fragment thereof that specifically binds to a biomarker of the present invention.

Another aspect of the present invention relates to a method for imaging melanoma in a patient, the method comprising administering to a patient an antibody or fragment thereof that specifically binds to a biomarker of the present invention.

Preferably, the antibody is conjugated to a detectable marker, for example a fluorescent marker or tag. Preferably, the antibody is a monoclonal antibody. Preferably, the antibody is conjugated to a growth inhibitory agent. Preferably, the antibody is conjugated to a cytotoxic agent, for example a toxin (e.g. immunotoxin), antibiotic, lytic enzyme or radioactive isotope.

Another aspect of the present invention relates to a composition comprising a biomarker of the present invention or an antibody or fragment thereof that binds to a biomarker of the present invention.

Preferably, the composition is a pharmaceutical composition.

Also provided by the present invention is a vaccine comprising a biomarker of the present invention or an antibody or fragment thereof that binds to a biomarker of the present invention.

Another aspect of the present invention relates to use of the melanoma specific biomarker, detectable in a body fluid or tissue, as a biomarker for melanoma.

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

Another aspect of the present invention relates to use of (i) a biomarker of the present invention, or (ii) an antibody or fragment thereof that specifically binds to a biomarker of the present invention, in the manufacture of a medicament for the treatment of melanoma.

Also provided is a composition comprising (i) a biomarker of the present invention, or (ii) an antibody or fragment thereof that specifically binds to a biomarker of the present invention, wherein the composition is for use in the treatment of melanoma.

Another aspect of the present invention relates to an antibody or fragment thereof that specifically binds to a biomarker of the present invention for use in a method of imaging melanoma in a patient.

In preferred embodiments, the methods and compositions of the invention are for treatment or diagnosis of disease at an early stage, for example, before symptoms of the disease appear.

In some embodiments, the methods and compositions of the invention are for treatment or diagnosis of disease at a clinical stage

According to another aspect of the present invention, there is provided a kit for use in the methods or uses described above, wherein the kit comprises a ligand capable of binding or specifically recognising the melanoma specific biomarker, detectable in a body fluid or tissue and reporter means.

Preferably, the kit is an array or chip.

Preferably, the kit comprises a microtitre plate, test strip, array or chip.

DETAILED DESCRIPTION OF THE INVENTION

Example embodiments of the present invention will now be described with reference to the accompanying figures.

Figure 1 shows section through a melanoma, normal tissue and normal tissue adjacent to a melanoma (NAT) core; tumour cells are stained using an anti-EN2 antibody;

Figure 2 shows FACS analysis of EN2 protein on the surface of the primary melanoma cell populations 'ML' and 'MK'. Plot A is a negative control with PE-tagged secondary antibody only. Plot B is with both primary (anti-EN2) and secondary antibody; the strong shift to the right indicates binding of the anti-EN2 antibody to the surface of the cells;

Figure 3 shows the nucleic acid sequence of EN2 (SEQ ID NO:1);

Figure 4 shows the amino acid sequence of EN2 (SEQ ID NO:2);

Figure 5 shows EN2 expression in melanoma. Representative examples of EN2 expression in melanoma compared to normal skin: A: Lymph node, metastatic malignant melanoma of right neck; B: Skin, malignant melanoma of right chest wall; C: Lymph node, metastatic malignant melanoma of armpit; D: Normal skin – no expression of EN2;

Figure 6 shows generation of EN2-specific CTL from melanoma patients. T cells from two melanoma patients (MEL02 and MEL04) were stimulated with pooled EN2 peptides five times before testing their specificity in a 51Cr-release cytotoxicity assay; and

Figure 7 shows EN2 vaccination delays tumor growth. Growth of K1735 melanoma tumors in C3H mice immunized with EN2 in alum (adjuvant: 'adj'), adjuvant alone or PBS.

The invention relates to melanoma specific biomarkers.

The world wide incidence of melanoma has been constantly increasing during the last years. Whilst surgical excision is effective when primary tumors are thin, at later stages of the disease patients often succumb due to failure of metastasis control. Several studies have now shown the existence of cell-mediated immunity in patients with advanced metastatic melanoma. Thus identifying and targeting clinically relevant antigens for immunotherapy offers a promising alternative strategy to treat metastatic melanoma patients. As discussed herein, we have identified one such promising target antigen, the homeobox transcription factor ENGRAILED 2 (EN2). EN2 is specifically involved in patterning the region that gives rise to the cerebellum but more recently has been shown to be a candidate oncogene in breast and prostate cancer. Having performed an immunohistochemical study on a high density malignant melanoma tissue array we have found that 60% and 57% of malignant melanomas and metastatic melanomas respectively express EN2 (Figure 5). This is in contrast to no expression of EN2 in normal skin or other normal tissues.

Within this specification, the terms “comprises” and “comprising” are interpreted to mean “includes, among other things”. These terms are not intended to be construed as “consists of only”.

Within this specification, the term “about” means plus or minus 20%, more preferably plus or minus 10%, even more preferably plus or minus 5%, most preferably plus or minus 2%.

As used herein, the term "therapeutically effective amount" means the amount of a composition which is required to reduce the severity of and/or ameliorate at least one condition or symptom which results from the disease in question.

Within this specification embodiments have been described in a way which enables a clear and concise specification to be written, but it is intended and will be appreciated that embodiments may be variously combined or separated without parting from the invention.

For clinical use, a compound according to the present invention or prodrug form thereof is formulated into a pharmaceutical formulation which is formulated to be compatible with its intended route of administration, for example for oral, rectal, parenteral or other modes of administration. Pharmaceutical formulations are usually prepared by mixing the active substance with a conventional pharmaceutically acceptable diluent or carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Examples of pharmaceutically acceptable diluents or carrier are water, gelatin, gum arabicum, lactose, microcrystalline cellulose, starch, sodium starch glycolate, calcium hydrogen phosphate, magnesium stearate, talcum, colloidal silicon dioxide, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated.

Such formulations may also contain other pharmacologically active agents, and conventional additives, such as stabilizers, wetting agents, emulsifiers, flavouring agents, buffers, and the like.

The formulations can be further prepared by known methods such as granulation, compression, microencapsulation, spray coating, etc. The formulations may be prepared by conventional methods in the dosage form of tablets, capsules, granules, powders, syrups, suspensions, suppositories or injections. Liquid formulations may be prepared by dissolving or suspending the active substance in water or other suitable vehicles. Tablets and granules may be coated in a conventional manner.

Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric

acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, 'chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum mono stearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a compound according to an embodiment of the invention) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable,

biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the

invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Within this specification, "identity," as it is known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Percentage identity can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988), all of which are incorporated herein by reference in their entirety. Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity are codified in publicly available computer programs. Preferred computer program methods to determine percentage identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984), which is incorporated herein by reference in its entirety), BLASTP, BLASTN, and FASTA (Atschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990), which is incorporated herein by reference in its entirety). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990), which is incorporated herein by reference in its entirety). As an illustration, by a

polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence of "SEQ ID NO: A" it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence of "SEQ ID NO: A." In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having an amino acid sequence having at least, for example, 95% identity to a reference amino acid sequence of "SEQ ID NO:B" is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of "SEQ ID NO: B." *In other words, to obtain a polypeptide having an amino acid* sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences encoding a receptor at least 50% homologous to each other typically remain hybridized to each other. The conditions can be such that sequences at least about 65%, at least about 70%, or at least about 75% or more homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N. Y. (1989), 6. 3.1-6.3.6,

which is incorporated herein by reference in its entirety. One example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. In one embodiment, an isolated receptor nucleic acid molecule that hybridizes under stringent conditions to the sequence of SEQ ID NO:1 corresponds to a naturally-occurring nucleic acid molecule. As used herein, a"naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e. g., encodes a natural protein).

Within this specification, "antibody or antibody fragment" refers to an antibody (for example IgG, IgM, IgA, IgD or IgE) or fragment (such as a Fab, F(ab')2, Fv, disulphide linked Fv, scFv, closed conformation multispecific antibody, disulphide-linked scFv, diabody) whether derived from any species naturally producing an antibody, or created by recombinant DNA technology; whether isolated from serum, B- cells, hybridomas, transfectomas, yeast or bacteria.

Within this specification, the term "treatment" means treatment of an existing disease and/or prophylactic treatment in order to prevent incidence of a disease. As such, the methods of the invention can be used for the treatment, prevention, inhibition of progression or delay in the onset of disease.

The term "biomarker" is used throughout the art and means a distinctive biological or biologically-derived indicator of a process, event or condition. In other words, a biomarker is indicative of a certain biological state, such as the presence of cancerous tissue. In some cases, different forms of biomarkers can be indicative of certain disease states but, without being bound by theory, it is thought that merely the presence of elevated levels of the biomarkers of the present invention in body fluids or tissue, is indicative of melanoma. Although it is not currently envisaged that different glycoforms, for instance, of the EN2 peptide, are secreted, these are nevertheless encompassed by the present invention. For instance, different glycoforms, such as altered glycoform structure or sugar content, may yet be determined for EN2, but these are encompassed and may even also be indicative of the progress of melanoma. Truncations, mutations, or deletions of, or ligations to, the EN2 peptide, or fragment thereof, are also envisaged.

As discussed above, it has surprisingly been found that there is a significant increase in expression of the EN2 gene in melanoma compared to normal tissue. Furthermore, EN2 is found in biological fluid, e.g. the blood or serum of patients with melanoma. It is thought that EN-2 may be secreted or may be detectable in body fluids due to leaking from damaged or dead cells. Such increased levels are indicative of both early stage and late stage melanoma. Whilst there is a significant rise between control or normal levels and early stage melanoma, there is also a very significant increase between early and late stage melanoma. Broadly, it is an advantage of the present invention that the substance and also the state of the cancer can be detected. This aids in the prognosis and provision of suitable therapies.

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

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

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

EXAMPLES

Immunohistochemical (ICH) detection of EN2 protein in melanoma

Our studies have shown that EN2 protein is present in melanoma but not in the surrounding normal tissue. This was achieved using a high density tissue array (Biomax Melanoma array, cat# ME2082) that contains different melanoma samples together with normal tissue adjacent to the melanoma (NAT), and normal skin tissue from other sites ('normal'). The results are summarised in Figure 1.

Core type	Number on array	number that stain for EN2
Melanoma	192	148 (77%)
NAT	8	0 (0%)
Normal	8	0 (0%)

EN2 is present on the surface of primary melanoma cells

Two primary cell populations derived from melanoma were assessed for the presence of EN2 protein on the surface of the cells using an anti-EN2 antibody and fluorescently activated cell sorting (FACS) using the fluorescent tag PE. This revealed that both groups of cells have a very high level of EN2 protein on the surface of the cell (Fig 2).

Methods

Enzymatic staining for EN2

1. Deparaffinize slides in three changes of 100% xylene for 5 minutes each
2. Wash twice in 100% ethanol
3. 20 mins in 0.3% Methanol/H202 (300mls methanol + 900ul H2O2)
4. Rehydrate in an ethanol series of 70% and 50%
5. Rinse slides in distilled water for 5mins
6. Incubate slides in boiling citrate buffer pH6.0 for 12mins*
7. Leave the slides to cool down for 2 hours

8. Wash in distilled water for 3mins

9. 2 washes of 3mins in PBS

10. Incubate sections for 15 minutes with 2.4% horse serum in PBS/BSA 1% in a moist chamber. (Blocking serum needs to be raised in the same species as the 2° Ab).

11. Incubate overnight with 1° antibody (Abcam goat- anti EN2) in moist chamber, room temp

12. 3 washes of 3mins in PBS.

13. Incubate sections for 30 minutes with diluted biotinylated "universal" secondary antibody.

14. 3 washes of 3mins in PBS

15. Incubate sections for 30 minutes with VECTASTAIN R.T.U. ABC Reagent.

16. 3 washes of 3mins in PBS

17. Incubate sections in peroxidase substrate solution until desired stain intensity develops

18. (10mins for each slide, using impact DAB solution)

19. Rinse sections in distilled water.

20. Counterstain, (Haematoxylin QS: 100µl/slide for max 45secs) and put slides back in running tap water

21. Dehydrated slide in series of alcohol (50%, 70%, 100%), Xylene 1, 2, 3, quick dips for each.

22. Mounted slides with VectaMount mounting medium and stored slides at room temp

*Microwave antigen retrieval with 0.01M citrate buffer pH6.0

1. Prepare 0.01M citrate buffer from stock solution: 1:10 dilution with distilled water

2. Measure pH and bring to pH6.0 using 0.1M citric acid

3. Pour 1L of citrate buffer into plastic container and microwave for 20 minutes High

4. Add sections in a rack to the boiling citrate buffer and microwave for a further 12 minutes

Making 0.3% H2O2:

300ml Methanol

900µl H2O2 (30%) Hydrogen Peroxidase

Making Peroxidase Substrate Serum from imPACT DAB:

1ml Diluent and 1 drop Chromogen

Generation of EN2-specific CTL from melanoma patients and EN2 vaccination

We have used a reverse immunology strategy to identify several immunogenic HLA-A2 restricted EN2 epitopes with which we were able to generate EN2-specific CTL responses from the blood of both HLA-A2 positive healthy control donors and melanoma patients. The results are shown in figure 6.

As the maximum immunotherapeutic potential is achieved by antigens that can elicit both a cell-mediated and humoral response, we screened the sera from a large cohort of melanoma patients for IgG autoantibodies to EN2 and compared this to control sera from healthy age matched donors with no history of cancer.

We have preliminary data showing a beneficial effect of EN2 vaccination in a mouse model of melanoma where vaccinated animals developed a much smaller tumor than controls (Figure 7).

The fact that these animals also had a positive recall antigen response to the vaccine shows that the tumor outcome may well be immune mediated. We conclude from these data that EN2 is a promising target for melanoma immune therapy.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications are covered by the appended claims.

CLAIMS

1. A melanoma specific biomarker comprising:-

(i) a nucleic acid sequence comprising SEQ ID NO:1, or a fragment or variant thereof, or a nucleic acid molecule which comprises said nucleic acid sequence; or

(ii) an amino acid sequence comprising SEQ ID NO:2, or a fragment or variant thereof, or an amino acid molecule which comprises said amino acid sequence.

2. A biomarker according to claim 1, wherein the fragments or variants thereof comprise:-

(i) a nucleic acid sequence that has at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% nucleic acid sequence identity with SEQ ID NO:1, a nucleic acid sequence that is hybridizable thereto under stringent conditions, and/or a nucleic acid sequence that is complementary thereto;

(ii) an amino acid sequence that has at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% amino acid sequence identity with SEQ ID NO:2, or

(iii) an amino acid sequence encoded by a nucleic acid sequence of (i).

3. A biomarker according to claim 1 or 2, wherein the fragments thereof comprise (i) at least four, preferably at least five, preferably at least six, preferably at least seven, preferably at least eight consecutive amino acids from SEQ ID NO:2 or (ii) a fragment of the nucleic acid sequence of SEQ ID NO:1 which encodes at least four, preferably at least five, preferably at least six, preferably at least seven, preferably at least eight consecutive amino acids from SEQ ID NO:2.

4. A biomarker according to any preceding claim, wherein the fragments or variants thereof are functional fragments or variants thereof.

5. A method for diagnosing melanoma in a patient or for identifying a patient at risk of developing melanoma, the method comprising:

(a) determining an amount of a melanoma specific biomarker according to any preceding claim in a sample obtained from a patient;

(b) comparing the amount of the determined melanoma specific biomarker in the sample from the patient to the amount of the melanoma specific biomarker in a normal control;

wherein a difference in the amount of the melanoma specific biomarker in the sample from the patient compared to the amount of the melanoma specific biomarker in the normal control is associated with the presence of melanoma or is associated with a risk of developing melanoma.

6. A method according to claim 5 for detecting early stage melanoma, wherein an increase between the control and the sample obtained from the patient is indicative of early stage melanoma.

7. A method according to claim 5 for detecting late stage melanoma wherein an increase between the control and the sample obtained from the patient is indicative of late stage melanoma.

8. A method for monitoring the progression of melanoma in a patient, the method comprising:

(a) determining an amount of a melanoma specific biomarker according to any of claims 1 to 4 in a sample obtained from a patient;

(b) comparing the amount of the determined melanoma specific biomarker in the sample from the patient to the amount of the melanoma specific biomarker in a normal control; and

(c) repeating steps (a) and (b) at two or more time intervals,

wherein an increase in the amount of the melanoma specific biomarker from the patient over time is associated with an increase in the progression of melanoma and a decrease in the amount of the melanoma specific biomarker from the patient over time is associated with a decrease in the progression of melanoma.

9. A method according to claim 8 for monitoring a change in stage of melanoma, wherein an increase, relative to an earlier stage sample or control is indicative of progression of the melanoma from an earlier stage to later stage of disease.

10. A method for monitoring the efficacy of a treatment for melanoma, comprising detecting and/or quantifying the presence of a melanoma specific biomarker according to any of claims 1 to 4 in a sample obtained from a patient.

11. A method according to any of claims 5 to 10, wherein the sample comprises biological fluid or tissue obtained from the patient.

12. A method according to claim 11, wherein the biological fluid or tissue comprises blood, serum, plasma and/or lymph fluid.

13. A method for treating a patient with melanoma, the method comprising administering to a patient a therapeutically effective amount of (i) a biomarker according to any of claims 1 to 4, or (ii) an antibody or fragment thereof that specifically binds to a biomarker according to any of claims 1 to 4.

14. A method according to claim 13, wherein the antibody is conjugated to a cytotoxic agent.

15. A method for imaging melanoma in a patient, the method comprising administering to a patient an antibody or fragment thereof that specifically binds to a biomarker according to any of claims 1 to 4.

16. A method according to any of claims 13 to 15, wherein the antibody is conjugated to a detectable marker.

17. A composition comprising a biomarker according to any of claims 1 to 4, or an antibody or fragment thereof that binds to a biomarker according to any of claims 1 to 4.

18. A pharmaceutical composition comprising a composition according to claim 17.

19. A melanoma vaccine comprising a biomarker according to any of claims 1 to 4.

20. Use of a melanoma specific biomarker according to any of claims 1 to 4, detectable in a body fluid or tissue, as a biomarker for melanoma.

21. Use of (i) a biomarker according to any of claims 1 to 4, or (ii) an antibody or fragment thereof that specifically binds to a biomarker according to any of claims 1 to 4, in the manufacture of a medicament for the treatment of melanoma.

22. A composition comprising (i) a biomarker according to any of claims 1 to 4, or (ii) an antibody or fragment thereof that specifically binds to a biomarker according to any of claims 1 to 4, wherein the composition is for use in the treatment of melanoma.

23. An antibody or fragment thereof that specifically binds to a biomarker according to any of claims 1 to 4, for use in a method of imaging melanoma in a patient.

24. A kit for use in a method according to any of claims 5 to 16 or use according to claim 20, wherein the kit comprises a ligand capable of binding or specifically recognising a melanoma specific biomarker according to any of claims 1 to 4, detectable in a body fluid or tissue and reporter means.

Melanoma Normal tissue Normal adjacent tissue

Fig 1

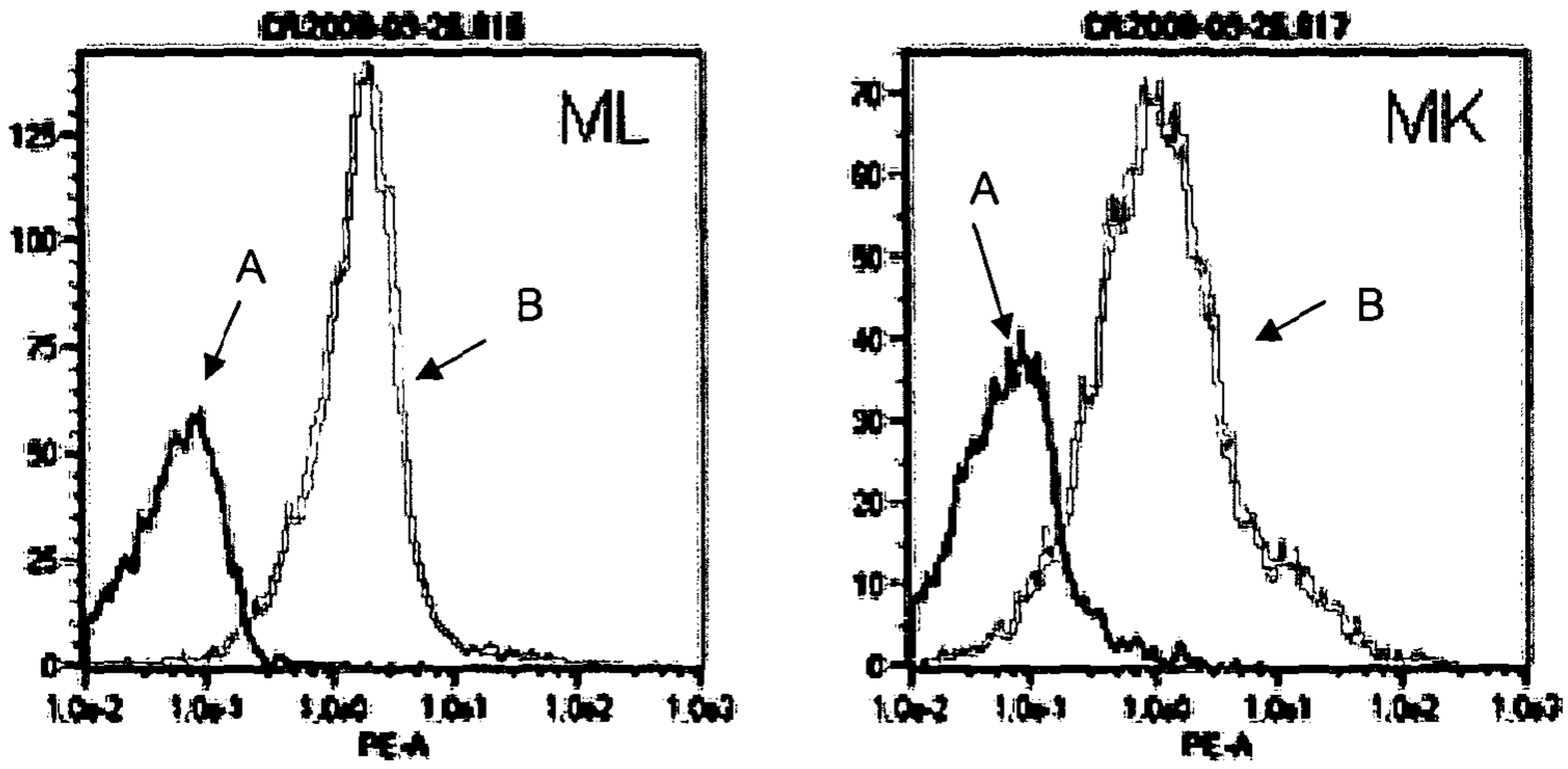

Fig 2

```
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3361 cattaaataa aacaaaatct ctttattata aaataaaaaa aaaaa
```

Fig 3

```
        10          20          30          40          50          60
MEENDPKPGE AAAAVEGQRQ PESSPGGGSG GGGGSSPGEA DTGRRRALML PAVLQAPGNH

        70          80          90         100         110         120
QHPHRITNFF IDNILRPEFG RRKDAGTCCA GAGGGRGGGA GGEGGASGAE GGGGAGGSEQ

       130         140         150         160         170         180
LLGSGSREPR QNPPCAPGAG GPLPAAGSDS PGDGEGGSKT LSLHGGAKKG GDPGGPLDGS

       190         200         210         220         230         240
LKARGLGGGD LSVSSDSDSS QAGANLGAQP MLWPAWVYCT RYSDRPSSGP RSRKPKKKNP

       250         260         270         280         290         300
NKEDKRPRTA FTAEQLQRLK AEFQTNRYLT EQRRQSLAQE LSLNESQIKI WFQNKRAKIK

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Fig 4

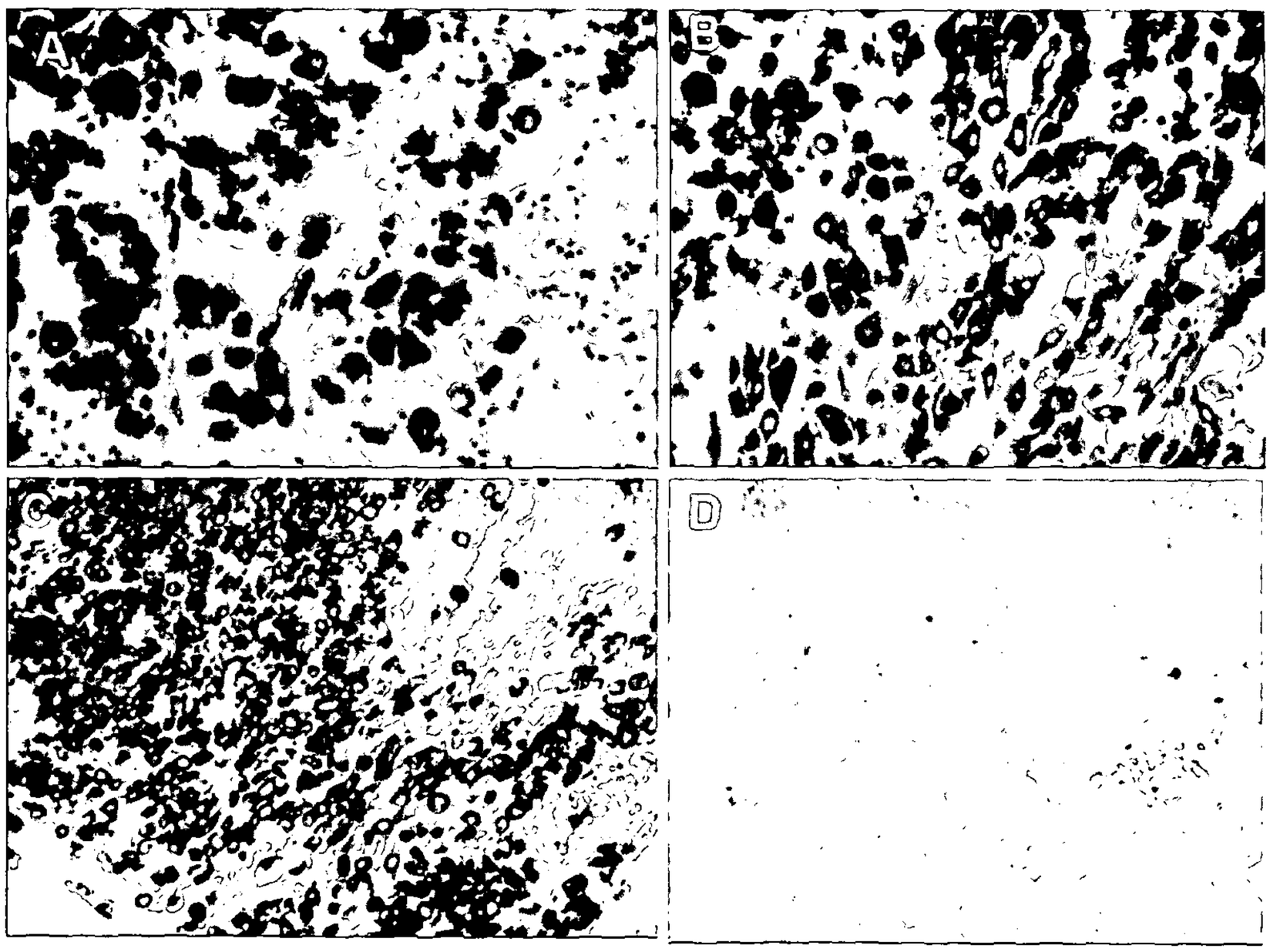
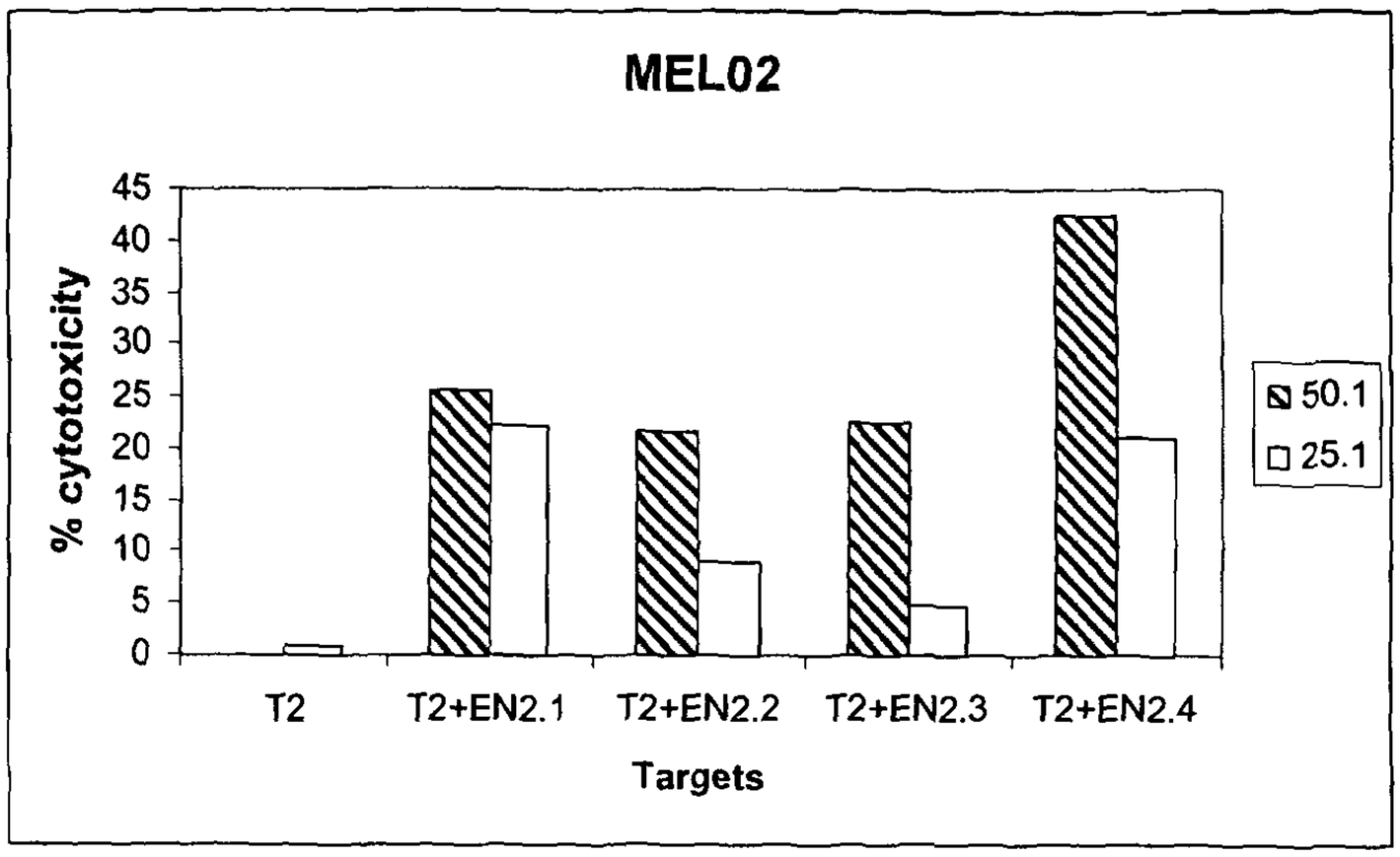


Fig 5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% cytotoxicity
45
40
35
30
25
20
15
10
5
0
T2
T2+EN2.1
T2+EN2.2
T2+EN2.3
T2+EN2.4
Targets
50.1
25.1

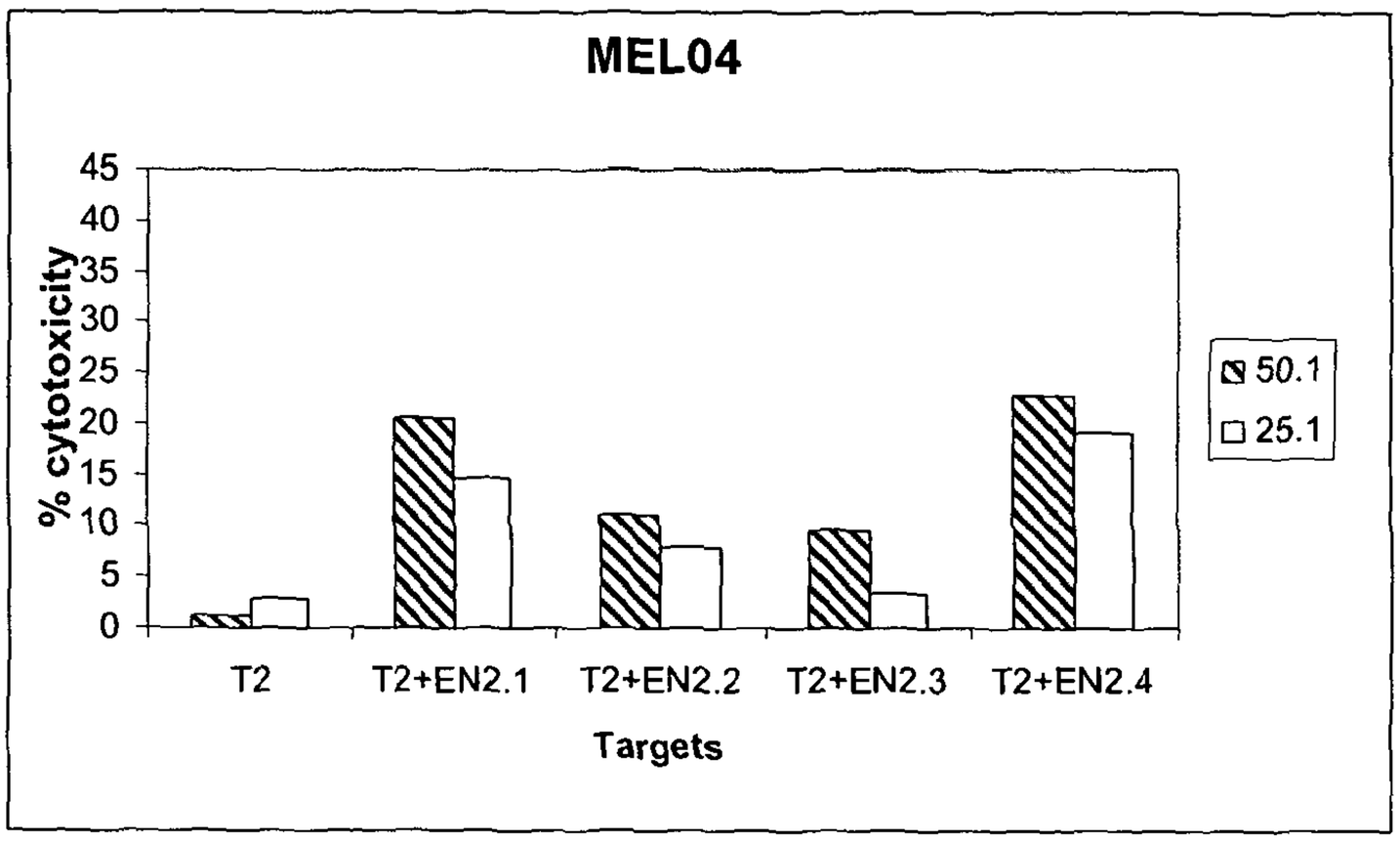
MEL04
% cytotoxicity
45
40
35
30
25
20
15
10
5
0
T2
T2+EN2.1
T2+EN2.2
T2+EN2.3
T2+EN2.4
Targets
50.1
25.1

Fig 6

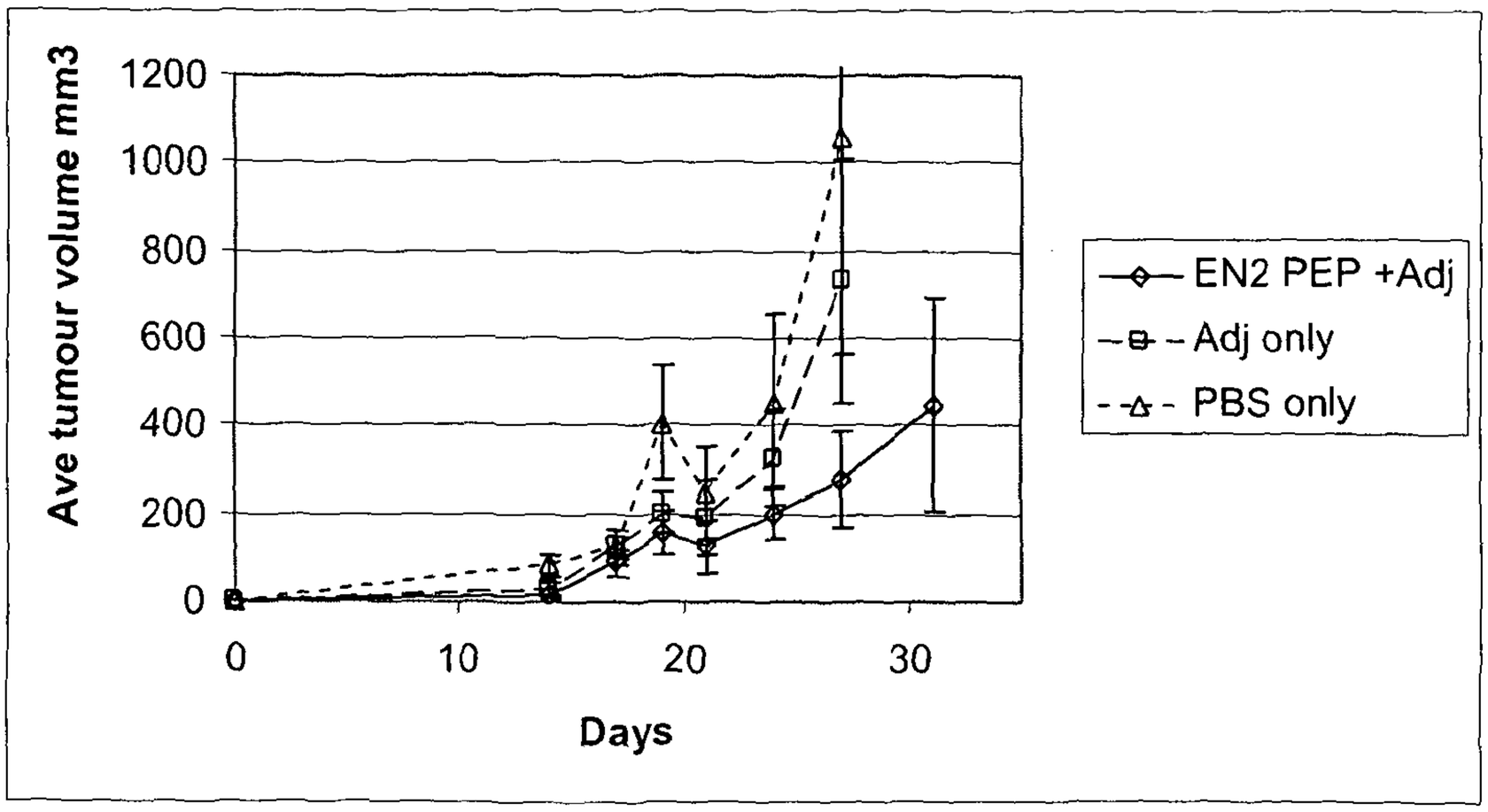
Ave tumour volume mm3
1200
1000
800
600
400
200
0
0
10
20
30
Days
EN2 PEP +Adj
Adj only
PBS only

Fig 7

Fig 1

Melanoma

Normal tissue

Normal adjacent tissue