



(51) International Patent Classification:
C12Q 1/68 (2018.01)

(21) International Application Number:
PCT/EP2018/059613

(22) International Filing Date:
16 April 2018 (16.04.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
17305448.7 14 April 2017 (14.04.2017) EP

(71) Applicants: INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE) [FR/FR]; 101 rue de Tolbiac, 75013 PARIS (FR). UNIVERSITE DE NICE SOPHIA ANTIPOLIS [FR/FR]; Grand Château Parc Valrose, 28 Avenue Valrose, 06103 NICE (FR). CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE [FR/FR]; 3 rue Michel Ange, 75016 PARIS (FR).

(72) Inventors: PAGES, Gilles; 37 boulevard du Larvotto, 98000 MONACO (MC). DOYEN, Jérôme; 2 bis rue Paul Bourget, 06100 NICE (FR). CLAREN MERCATI, Audrey; 2 Boulevard de Cimiez, Le Grand Palais entrée 6, 06000 NICE (FR). LUPU PLESU, Marilena; 72 Ancien Chemin de la Lanterne, Résidence Les Pierres Blanches, Bâtiment 1, Escalier B, 06200 NICE (FR).

(74) Agent: CABINET BECKER ET ASSOCIES; 25, rue Louis le Grand, 75002 PARIS (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF CANCER

(57) Abstract: The invention relates to a method for predicting the outcome of a cancer in patient afflicted with solid cancer after radiotherapy. The inventors investigated the comparative biological effects of proton (P) versus photon (X) radiation in head and neck squamous cell carcinoma (FiNSCC) cells. The inventors demonstrated that P and X radiation induced VEGF-C over-expression at both gene and protein levels in FiNSCC cells and in MDB cells and that VEGF-C is a major factor responsible for post-irradiation disease progression in FiNSCC patients, via promotion of lymphangiogenesis. The inventors demonstrated that VEGF-C is an important therapeutic target for FiNSCC patients who relapse after radiotherapy. The inventors investigated the presence of lymphatic markers in biopsies from primary and locally relapsed human FiNSCC, after X radiotherapy. The patients presented increased VEGF-C, bringing evidence that X radiotherapy promote lymphangiogenesis. Thus, the invention relates to a method for predicting the outcome of a cancer in patient afflicted with solid cancer after radiotherapy, comprising the step of determining the expression level of VEGF-C. The invention also relates to a VEGF-C inhibitor for use in the treatment of solid cancer in a patient classified as having a poor prognosis by the method of the invention.

METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF CANCER

FIELD OF THE INVENTION:

5 The present invention relates to methods and pharmaceutical compositions for the treatment of cancer.

BACKGROUND OF THE INVENTION:

Approximately 50% of all cancer patients are subject to radiotherapy during the course of their illness with an estimation that radiotherapy contributes to approximately 40% towards
10 curative treatment (1). The goal of radiotherapy is to deliver loco-regionally a specific dose of radioactivity that will allow the destruction of cancer cells, while limiting the exposure of surrounding healthy tissues. Among the ionizing radiation treatments, the large majority consists of photons (X) of high energy (5-20 MeV). However, the main disadvantage of X radiotherapy is represented by the deposition of radiation also at the level of surrounding
15 healthy tissues, leading to side effects. Although the ionizing radiation by proton beams (P) is currently more expensive and more difficult to produce, it has the physical advantage of delivering no radiation outside of the intended targeted area, thanks to the so-called Bragg peak (2).

P radiotherapy is mainly proposed for the treatment of uveal melanoma, skull base and
20 paraspinal tumors due to its high precision in tumor targeting with a very high irradiation dose next to radiosensitive structures (2). It is also proposed for the pediatric tumors based on the advantage to deliver a much lower integral dose, which significantly reduces the risk of radiation induced cancers in a long-life expectancy setting (2). Several retrospective and dosimetry studies have suggested an advantage of P radiotherapy in other tumors located near
25 organs at risk, such as the head and neck squamous cell carcinoma (HNSCC) (2).

The head and neck cancers are among the ten most common types of cancer and the
7th cause of mortality from cancer worldwide. Depending on disease stage, the treatment of HNSCC consists of either chemoradiotherapy and/or surgical excision (3). However, conventional radiotherapy with X in HNSCC remains difficult, due to the proximity of
30 numerous organs at risk (i.e. salivary glands, esophagus and larynx). Recent studies have shown an advantage of P, over X radiotherapy, in inducing lower toxicities (4) and lower dose delivery to organs of risk (5) in HNSCC patients.

Despite of the currently available therapeutic strategies, the five-year overall survival rate of HNSCC patients is only 53% (6) because of a high percentage of a poor response to

therapy and high recurrence rates. Sentinel lymph node metastasis, the first sign of tumor progression, was directly correlated to prognosis in HNSCC patients (7). Vascular Endothelial Growth Factor C (VEGF-C) is a major pro-lymphangiogenic factor responsible for the metastatic dissemination of cancer cells (8). A significant correlation has been observed
5 between intra-tumor lymphatic vessel density and lymph node metastasis in patients with HNSCC (9). Moreover, VEGF-C expression levels correlated with lymphatic vessel density and lymph node metastasis in these patients (10, 11). VEGF-C-dependent development of the lymphatic network might also be the major route of spread of tumor cells when the patients become resistant to therapy (8).

10 Beside the physical advantage of P vs. X irradiation and the RBE, few comparative preclinical studies have been conducted that contrast cellular/biological response to P vs X radiations (12-15).

P irradiation led to distinct gene and protein expression profiles (12). Mice receiving total-body irradiation with either P or X had enhanced plasma levels of transforming growth
15 factor- β , only after X irradiation (13). Moreover, X irradiation promoted angiogenesis, thus enhancing metastasis by up-regulation of various pro-angiogenic factors (14). By contrast, low dose P irradiation did not induce the pro-angiogenic and pro-inflammatory genes, impaired tumor cell invasion *in vitro*, and attenuated tumor growth rate in mice (14). By down-regulating integrins and matrix-metalloproteinases (MMP), P irradiation also reduced
20 invasive and migratory properties of tumor cells (15).

Therefore, beside the physical advantage in dose deposition, P may have different biological properties, as compared to X radiation at a similar dose. The purpose of the present study was thus to analyze the different biological behaviors of HNSCC cells when exposed to P vs X radiation. The study focused on molecules with key roles in the progression and
25 prognosis of HNSCC, such as the inflammatory cytokines: Interleukin 6 (IL6) (16), Interleukin 8 (IL8) (17); (lymph)angiogenic factors: Vascular Endothelial Grow Factors (VEGF) A, C and D and their receptors: Vascular Endothelial Growth Factor Receptor (VEGFR) 1, 2 and 3, Neuropilin (NRP) 1 and 2 (18, 19); factors involved in lymphatic vessels development: lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), prospero
30 homeobox 1 (PROX1) transcription factor, and podoplanin (PDPN), a mucin-type transmembrane protein (20); pro-inflammatory chemokine C-C Motif Chemokine Ligand 2 (CCL2) involved in cell migration (21); cell cycle regulators: polo-like kinase 1 (PLK1) (22) and telomeric repeat-binding factor 2 (TRF2) transcription factor (23); immune checkpoint

molecule programmed death-ligand 1 (PD-L1) involved in anergy and tumor progression (24).

The role of the above-mentioned molecules in the post-irradiation progression of HNSCC has not been elucidated. Our working hypothesis was that different radiation types would lead to different intrinsic and extrinsic biological responses, allowing the adaptation of tumor cells. Therefore, we studied the impacts of P vs X irradiation on human HNSCC cells viability; proliferation; whole transcriptome profile and expression of key genes/proteins implicated in (lymph)angiogenesis/metastasis, inflammation, tumor cell proliferation and anti-tumor immunity; tumorigenic potential, and depicted the molecular mechanisms of post-irradiation VEGF-C regulation, to set the basis for improved therapeutic approaches for HNSCC.

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of cancer.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors investigated the comparative biological effects of P versus X radiation in head and neck squamous cell carcinoma (HNSCC) cells by assessing the relative biological effectiveness (RBE), viability, proliferation and mRNA levels for genes involved in (lymph)angiogenesis, inflammation, proliferation and anti-tumor immunity. These parameters, particularly VEGF-C protein levels and regulations, were documented in freshly irradiated and/or long-term surviving cells receiving low/high-dose, single (SI)/multiple (MI) irradiations with P/X. The RBE was found to be 1.1 Key (lymph)angiogenesis and inflammation genes were down-regulated, except for vegf-c, after P and up-regulated after X irradiation in MI surviving cells, demonstrating a more favorable profile after P irradiation. Both irradiation types stimulated vegf-c promoter activity in a NF- κ B-dependent transcriptional regulation manner, but at a lesser extent after P, as compared to X irradiation, which correlated with mRNA and protein levels. The cells surviving to MI by P or X generated tumors with higher volume, anarchic architecture and increased density of blood vessels. Increased lymphangiogenesis and a transcriptomic analysis in favor of a more aggressive phenotype were observed in tumors generated with X-irradiated cells. Increased detection of lymphatic vessels in relapsed tumors from patients receiving X radiotherapy was consistent with these findings. The present invention demonstrates the biological advantage of P, as compared to X irradiation. In addition to its physical advantage in dose deposition, P irradiation may help to improve treatment approaches for HNSCC.

The inventors demonstrated that P and X radiation-induced VEGF-C over-expression at both gene and protein levels in HNSCC cells and in medulloblastoma (MDB) cells and that VEGF-C is a major factor responsible for post-irradiation disease progression in HNSCC patients, via promotion of lymphangiogenesis. The inventors also demonstrated that VEGF-C is an important therapeutic target for HNSCC patients who relapse after radiotherapy with either P or X. The inventors also investigated the presence of lymphatic markers in biopsies from primary and locally relapsed human HNSCC, after conventional X radiotherapy. The cohort of patients presented increased protein and/or mRNA levels of VEGF-C, PDPN, LYVE1 and PROX1, bringing evidence that X radiotherapy may promote lymphangiogenesis.

Accordingly, the present invention relates to a method for predicting the outcome of a cancer in patient afflicted with solid cancer after radiotherapy treatment, comprising the steps of: i) determining the expression level of VEGF-C in a biological sample obtained from said patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient has a good prognosis when the level determined at step i) is lower than the predetermined reference value or concluding that the patient has a poor prognosis when the level determined at step i) is higher than the predetermined reference value.

As used herein, the term “patient” denotes a mammal. Typically, a patient according to the invention refers to any patient (preferably human) afflicted with solid cancer. The term “patient” also refers to any patient afflicted with head and neck squamous cell carcinoma (HNSCC) or with medulloblastoma (MDB) for example. The term “patient” also refers to any patient afflicted with solid cancer receiving radiotherapy. The term “patient” also refers to any patient afflicted with head and neck squamous cell carcinoma (HNSCC) or with medulloblastoma (MDB) receiving radiotherapy.

The term “solid cancer” has its general meaning in the art and refers to solid cancer selected from the group consisting of, but not limited to, head and neck squamous cell carcinoma (HNSCC), adrenal cortical cancer, anal cancer, bile duct cancer (e.g. periphilar cancer, distal bile duct cancer, intrahepatic bile duct cancer), bladder cancer, bone cancer (e.g. osteoblastoma, osteochondroma, hemangioma, chondromyxoid fibroma, osteosarcoma, chondrosarcoma, fibrosarcoma, malignant fibrous histiocytoma, giant cell tumor of the bone, chordoma, multiple myeloma), brain and central nervous system cancer (e.g. meningioma, astocytoma, oligodendrogliomas, ependymoma, gliomas, medulloblastoma (MDB), ganglioglioma, Schwannoma, germinoma, craniopharyngioma), breast cancer (e.g. ductal carcinoma in situ, infiltrating ductal carcinoma, infiltrating lobular carcinoma, lobular

carcinoma in situ, gynecomastia), cervical cancer, colorectal cancer, endometrial cancer (e.g. endometrial adenocarcinoma, adenocanthoma, papillary serous adenocarcinoma, clear cell), esophagus cancer, gallbladder cancer (mucinous adenocarcinoma, small cell carcinoma), gastrointestinal carcinoid tumors (e.g. choriocarcinoma, chorioadenoma destruens), Kaposi's
5 sarcoma, kidney cancer (e.g. renal cell cancer), laryngeal and hypopharyngeal cancer, liver cancer (e.g. hemangioma, hepatic adenoma, focal nodular hyperplasia, hepatocellular carcinoma), lung cancer (e.g. small cell lung cancer, non-small cell lung cancer), mesothelioma, plasmacytoma, nasal cavity and paranasal sinus cancer (e.g. esthesioneuroblastoma, midline granuloma), nasopharyngeal cancer, neuroblastoma, oral
10 cavity and oropharyngeal cancer, ovarian cancer, pancreatic cancer, penile cancer, pituitary cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma (e.g. embryonal rhabdomyosarcoma, alveolar rhabdomyosarcoma, pleomorphic rhabdomyosarcoma), salivary gland cancer, skin cancer (e.g. melanoma, nonmelanoma skin cancer), stomach cancer, testicular cancer (e.g. seminoma, nonseminoma germ cell cancer), thymus cancer, thyroid
15 cancer (e.g. follicular carcinoma, anaplastic carcinoma, poorly differentiated carcinoma, medullary thyroid carcinoma), vaginal cancer, vulvar cancer, and uterine cancer (e.g. uterine leiomyosarcoma).

In some embodiment, the cancer is head and neck squamous cell carcinoma (HNSCC).

In some embodiment, the cancer is medulloblastoma (MDB).

20 The term “biological sample” refers to any biological sample derived from the patient such as solid cancer sample, biopsy sample, blood sample, plasma sample, or serum sample. Said biological sample is obtained for the purpose of the *in vitro* evaluation.

The term “radiotherapy treatment” has its general meaning in the art and refers to photon (X) radiotherapy and proton (P) radiotherapy. The term “radiotherapy treatment” also
25 refers to photon irradiation and proton irradiation. The term “radiotherapy treatment” also refers to single irradiation (SI) and multiple irradiations (MI) such as described in the example. The term “radiotherapy treatment” also refers to radiation therapy using radiotherapeutic agent administered to the patient afflicted with solid cancer.

Radiation therapy or radiotherapy is the medical use of irradiation (i.e. ionizing
30 radiation) as part of cancer treatment to control malignant cells. Ionizing radiation deposits energy that injures or destroys cells in the area being treated (the target tissue) by damaging their genetic material, making it impossible for these cells to continue to grow. One type of radiation therapy commonly used involves photons, e.g. X-rays. Depending on the amount of

energy they possess, the rays can be used to destroy cancer cells on the surface of or deeper in the body. The higher the energy of the x-ray beam, the deeper the x-rays can go into the target tissue. Linear accelerators and betatrons produce x-rays of increasingly greater energy. The use of machines to focus radiation (such as x-rays) on a cancer site is called external beam radiotherapy. Gamma rays are another form of photons used in radiotherapy. Gamma rays are produced spontaneously as certain elements (such as radium, uranium, and cobalt 60) release radiation as they decompose, or decay. Another technique for delivering radiation to cancer cells is to place radioactive implants directly in a tumor or body cavity. This is called internal radiotherapy. Brachytherapy, interstitial irradiation, and intracavitary irradiation are types of internal radiotherapy. In this treatment, the radiation dose is concentrated in a small area. A further technique is intra-operative irradiation, in which a large dose of external radiation is directed at the tumor and surrounding tissue during surgery.

Another approach is particle beam radiation therapy. This type of therapy differs from photon radiotherapy in that it involves the use of fast-moving subatomic particles to treat localized cancers. Some particles (protons, neutrons, pions, and heavy ions) deposit more energy along the path they take through tissue than do x-rays or gamma rays, thus causing more damage to the cells they hit. This type of radiation is often referred to as high linear energy transfer (high LET) radiation. Radio-sensitizers make the tumor cells more likely to be damaged, and radio-protectors protect normal tissues from the effects of radiation.

A person of ordinary skill in the radiotherapy art knows how to determine an appropriate dosing and application schedule, depending on the nature of the disease and the constitution of the patient. In particular, the person knows how to assess dose-limiting toxicity (DLT) and how to determine the maximum tolerated dose (MTD) accordingly. More particularly, the amount of radiation used in photon radiation therapy is measured in gray (Gy), and varies depending on the type and stage of cancer being treated. Many other factors are considered by radiation oncologists when selecting a dose, including whether the patient is receiving chemotherapy, patient co-morbidities, whether radiation therapy is being administered before or after surgery, and the degree of success of surgery. Moreover, the total dose is often fractionated. Fractionation regimes are individualized between different radiotherapy centers. The typical fractionation schedule for adults is 1 to 2 Gy per day, five days a week. In some cases, two fractions per day are used near the end of a course of treatment. This schedule is known as a concomitant boost regimen or hyperfractionation. Thus, in another preferred embodiment, the radiotherapy can be applied at a dose in a range

from about 1 to 80Gy, about 10 to 55Gy, preferably from about 15 to 50 Gy, such as 20 to 40Gy, concretely from about 20 to 35 Gy, and more concretely from about 25 to 30 Gy.

As used herein, the term “VEGF-C” has its general meaning in the art and refers to Vascular Endothelial Growth Factor C, a member of the vascular endothelial growth factor family.

As used herein, the term “Good Prognosis” refers to a patient afflicted with solid cancer receiving radiotherapy treatment that is likely to not present lymph node metastasis, and/or that is likely to not present cancer relapse, and/or that is likely to present a high overall survival (OS), event-free survival (EFS), and/or metastasis-free survival (MFS).

As used herein, the term “Poor Prognosis” or “Bad Prognosis” refers to a patient afflicted with solid cancer receiving radiotherapy treatment that is likely to present lymph node metastasis, and/or that is likely to present cancer relapse, and/or that is likely to present short overall survival (OS), progression free survival (PFS) and/or metastasis.

In some embodiment, the method of the invention also comprises determining the expression level of PDPN, LYVE1 and PROX1.

The term “PDPN” has its general meaning in the art and refers to podoplanin, a mucin-type transmembrane protein.

The term “LYVE1” has its general meaning in the art and refers to lymphatic vessel endothelial hyaluronan receptor 1.

The term “PROX1” has its general meaning in the art and refers to prospero homeobox 1 transcription factor.

As used herein, the “reference value” refers to a threshold value or a cut-off value. The setting of a single “reference value” thus allows discrimination between a poor and a good prognosis with respect to the lymph node metastasis, cancer relapse and overall survival (OS) for a patient. Typically, a “threshold value” or “cut-off value” can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. Preferably, the

person skilled in the art may compare the expression level (obtained according to the method of the invention) with a defined threshold value. In one embodiment of the present invention, the threshold value is derived from the expression level (or ratio, or score) determined in a biological sample derived from one or more patients having solid cancer. Furthermore, retrospective measurement of the expression level (or ratio, or scores) in properly banked historical patient samples may be used in establishing these threshold values.

Predetermined reference values used for comparison may comprise “cut-off” or “threshold” values that may be determined as described herein. Each reference (“cut-off”) value for the biomarker of interest may be predetermined by carrying out a method comprising the steps of

- a) providing a collection of samples from patients suffering of solid cancer;
- b) determining the expression level of the biomarker for each sample contained in the collection provided at step a);
- c) ranking the tumor tissue samples according to said expression level;
- d) classifying said samples in pairs of subsets of increasing, respectively decreasing, number of members ranked according to their expression level,
- e) providing, for each sample provided at step a), information relating to the responsiveness of the patient or the actual clinical outcome for the corresponding cancer patient (i.e. the duration of the event-free survival (EFS), metastasis-free survival (MFS) or the overall survival (OS) or both);
- f) for each pair of subsets of samples, obtaining a Kaplan Meier percentage of survival curve;
- g) for each pair of subsets of samples calculating the statistical significance (p value) between both subsets;
- h) selecting as reference value for the expression level, the value of expression level for which the p value is the smallest.

For example the expression level of a biomarker has been assessed for 100 cancer samples of 100 patients. The 100 samples are ranked according to their expression level. Sample 1 has the best expression level and sample 100 has the worst expression level. A first grouping provides two subsets: on one side sample Nr 1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side the 98 remaining samples etc., until the last grouping: on one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for

the corresponding cancer patient, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated.

5 The reference value is selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the expression level corresponding to the boundary between both subsets for which the p value is minimum is considered as the reference value. It should be noted that the reference value is not necessarily the median value of expression levels.

10 In routine work, the reference value (cut-off value) may be used in the present method to discriminate cancer samples and therefore the corresponding patients.

Kaplan–Meier curves of percentage of survival as a function of time are commonly to measure the fraction of patients living for a certain amount of time after treatment and are well known by the man skilled in the art.

15 The man skilled in the art also understands that the same technique of assessment of the expression level of a biomarker should of course be used for obtaining the reference value and thereafter for assessment of the expression level of a biomarker of a patient subjected to the method of the invention.

20 In one embodiment, the reference value may correspond to the expression level of the biomarker determined in a sample associated having a good prognosis. Accordingly, a higher expression level of the biomarker than the reference value is indicative of a patient having poor prognosis, and a lower or equal expression level of the biomarker than the reference value is indicative of a patient having a good prognosis.

25 In another embodiment, the reference value may correspond to the expression level of the biomarker determined in a sample associated with a patient having a poor prognosis. Accordingly, a higher or equal expression level of the biomarker than the reference value is indicative of a patient having poor prognosis, and a lower expression level of the biomarker than the reference value is indicative of a patient having good prognosis.

30 In another embodiment, a score which is a composite of the expression levels of the different biomarkers may also be determined and compared to a reference value wherein a difference between said score and said reference value is indicative of a patient having a good or poor prognosis

In a particular embodiment, the score may be generated by a computer program.

Analyzing the biomarker expression level may be assessed by any of a wide variety of well-known methods for detecting expression of a transcribed nucleic acid or translated protein.

5 In one embodiment, the biomarker expression level is assessed by analyzing the expression of the protein translated from said gene. Said analysis can be assessed using an antibody (e.g., a radio-labeled, chromophore-labeled, fluorophore-labeled, or enzyme-labeled antibody), an antibody derivative (e.g., an antibody conjugate with a substrate or with the protein or ligand of a protein of a protein/ligand pair (e.g., biotin-streptavidin)), or an antibody fragment (e.g., a single-chain antibody, an isolated antibody hypervariable domain, 10 etc.) which binds specifically to the protein translated from the gene encoding for the biomarker.

Methods for measuring the expression level of a biomarker in a sample may be assessed by any of a wide variety of well-known methods from one of skill in the art for detecting expression of a protein including, but not limited to, direct methods like mass 15 spectrometry-based quantification methods, protein microarray methods, enzyme immunoassay (EIA), radioimmunoassay (RIA), Immunohistochemistry (IHC), Western blot analysis, ELISA, Luminex, ELISPOT and enzyme linked immunoabsorbant assay and indirect methods based on detecting expression of corresponding messenger ribonucleic acids (mRNAs). The mRNA expression profile may be determined by any technology known by a 20 man skilled in the art. In particular, each mRNA expression level may be measured using any technology known by a man skilled in the art, including nucleic microarrays, quantitative Polymerase Chain Reaction (qPCR), next generation sequencing and hybridization with a labelled probe.

Said direct analysis can be assessed by contacting the sample with a binding partner 25 capable of selectively interacting with the biomarker present in the sample. The binding partner may be an antibody that may be polyclonal or monoclonal, preferably monoclonal (e.g., a isotope-label, element-label, radio-labeled, chromophore-labeled, fluorophore-labeled, or enzyme-labeled antibody), an antibody derivative (e.g., an antibody conjugate with a substrate or with the protein or ligand of a protein of a protein/ligand pair (e.g., biotin-streptavidin)), or an antibody fragment (e.g., a single-chain antibody, an isolated antibody hypervariable domain, etc.) which binds specifically to the protein translated from the gene 30 encoding for the biomarker of the invention. In another embodiment, the binding partner may be an aptamer.

The binding partners of the invention such as antibodies or aptamers may be labelled with a detectable molecule or substance, such as an isotope, an element, a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

5 As used herein, the term "labelled", with regard to the antibody, is intended to encompass direct labelling of the antibody or aptamer by coupling (i.e., physically linking) a detectable substance, such as an isotope, an element, a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the antibody or aptamer, as well as indirect labelling of the probe or antibody by reactivity with a
10 detectable substance. An antibody or aptamer of the invention may be produced with a specific isotope or a radioactive molecule by any method known in the art. For example radioactive molecules include but are not limited to radioactive atom for scintigraphic studies such as I123, I124, In111, Re186, Re188, specific isotopes include but are not limited to ¹³C, ¹⁵N, ¹²⁶I, ⁷⁹Br, ⁸¹Br.

15 The above mentioned assays generally involve the binding of the binding partner (ie. antibody or aptamer) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated
20 beads, magnetically responsive beads, silicon wafers.

In a particular embodiment, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize said biomarker. A sample containing or suspected of containing said biomarker is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the
25 plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art such as Singulex, Quanterix, MSD, Bioscale, Cytof.

30 In one embodiment, an Enzyme-linked immunospot (ELISpot) method may be used. Typically, the sample is transferred to a plate which has been coated with the desired anti-biomarker capture antibodies. Revelation is carried out with biotinylated secondary Abs and

standard colorimetric or fluorimetric detection methods such as streptavidin-alkaline phosphatase and NBT-BCIP and the spots counted.

In one embodiment, when multi-biomarker expression measurement is required, use of beads bearing binding partners of interest may be preferred. In a particular embodiment, the
5 bead may be a cytometric bead for use in flow cytometry. Such beads may for example correspond to BD™ Cytometric Beads commercialized by BD Biosciences (San Jose, California). Typically cytometric beads may be suitable for preparing a multiplexed bead assay. A multiplexed bead assay, such as, for example, the BD(TM) Cytometric Bead Array, is a series of spectrally discrete beads that can be used to capture and quantify soluble
10 antigens. Typically, beads are labelled with one or more spectrally distinct fluorescent dyes, and detection is carried out using a multiplicity of photodetectors, one for each distinct dye to be detected. A number of methods of making and using sets of distinguishable beads have been described in the literature. These include beads distinguishable by size, wherein each size bead is coated with a different target-specific antibody (see e.g. Fulwyler and McHugh,
15 1990, Methods in Cell Biology 33:613-629), beads with two or more fluorescent dyes at varying concentrations, wherein the beads are identified by the levels of fluorescence dyes (see e.g. European Patent No. 0 126,450), and beads distinguishably labelled with two different dyes, wherein the beads are identified by separately measuring the fluorescence intensity of each of the dyes (see e.g. U.S. patent Nos. 4,499,052 and 4,717,655). Both one-
20 dimensional and two-dimensional arrays for the simultaneous analysis of multiple antigens by flow cytometry are available commercially. Examples of one-dimensional arrays of singly dyed beads distinguishable by the level of fluorescence intensity include the BD(TM) Cytometric Bead Array (CBA) (BD Biosciences, San Jose, Calif.) and Cyto-Plex(TM) Flow Cytometry microspheres (Duke Scientific, Palo Alto, Calif.). An example of a two-
25 dimensional array of beads distinguishable by a combination of fluorescence intensity (five levels) and size (two sizes) is the QuantumPlex(TM) microspheres (Bangs Laboratories, Fisher, Ind.). An example of a two-dimensional array of doubly-dyed beads distinguishable by the levels of fluorescence of each of the two dyes is described in Fulton et al. (1997, Clinical Chemistry 43(9):1749-1756). The beads may be labelled with any fluorescent
30 compound known in the art such as e.g. FITC (FL1), PE (FL2), fluorophores for use in the blue laser (e.g. PerCP, PE-Cy7, PE-Cy5, FL3 and APC or Cy5, FL4), fluorophores for use in the red, violet or UV laser (e.g. Pacific blue, pacific orange). In another particular embodiment, bead is a magnetic bead for use in magnetic separation. Magnetic beads are known to those of skill in the art. Typically, the magnetic bead is preferably made of a

magnetic material selected from the group consisting of metals (e.g. ferrum, cobalt and nickel), an alloy thereof and an oxide thereof. In another particular embodiment, bead is bead that is dyed and magnetized.

In one embodiment, protein microarray methods may be used. Typically, at least one
5 antibody or aptamer directed against the biomarker is immobilized or grafted to an array(s), a solid or semi-solid surface(s). A sample containing or suspected of containing the biomarker is then labelled with at least one isotope or one element or one fluorophore or one colorimetric tag that are not naturally contained in the tested sample. After a period of incubation of said sample with the array sufficient to allow the formation of antibody-antigen
10 complexes, the array is then washed and dried. After all, quantifying said biomarker may be achieved using any appropriate microarray scanner like fluorescence scanner, colorimetric scanner, SIMS (secondary ions mass spectrometry) scanner, maldi scanner, electromagnetic scanner or any technique allowing quantifying said labels.

In another embodiment, the antibody or aptamer grafted on the array is labelled.

15 In another embodiment, reverse phase arrays may be used. Typically, at least one sample is immobilized or grafted to an array(s), a solid or semi-solid surface(s). An antibody or aptamer against the suspected biomarker is then labelled with at least one isotope or one element or one fluorophore or one colorimetric tag that is not naturally contained in the tested sample. After a period of incubation of said antibody or aptamer with the array sufficient to
20 allow the formation of antibody-antigen complexes, the array is then washed and dried. After all, detecting quantifying and counting by D-SIMS said biomarker containing said isotope or group of isotopes, and a reference natural element, and then calculating the isotopic ratio between the biomarker and the reference natural element. may be achieve using any appropriate microarray scanner like fluorescence scanner, colorimetric scanner, SIMS
25 (secondary ions mass spectrometry) scanner, maldi scanner, electromagnetic scanner or any technique allowing to quantify said labels.

In one embodiment, said direct analysis can also be assessed by mass Spectrometry. Mass spectrometry-based quantification methods may be performed using either labelled or unlabelled approaches (DeSouza and Siu, 2012). Mass spectrometry-based quantification
30 methods may be performed using chemical labeling, metabolic labeling or proteolytic labeling. Mass spectrometry-based quantification methods may be performed using mass spectrometry label free quantification, LTQ Orbitrap Velos, LTQ-MS/MS, a quantification based on extracted ion chromatogram EIC (progenesis LC-MS, Liquid chromatography-mass

spectrometry) and then profile alignment to determine differential expression of the biomarker.

In another embodiment, the biomarker expression level is assessed by analyzing the expression of mRNA transcript or mRNA precursors, such as nascent RNA, of biomarker gene. Said analysis can be assessed by preparing mRNA/cDNA from cells in a sample from a patient, and hybridizing the mRNA/cDNA with a reference polynucleotide. The prepared mRNA/cDNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses, such as quantitative PCR (TaqMan), and probes arrays such as GeneChip(TM) DNA Arrays (AFFYMETRIX).

Advantageously, the analysis of the expression level of mRNA transcribed from the gene encoding for biomarkers involves the process of nucleic acid amplification, e. g., by RT-PCR (the experimental embodiment set forth in U. S. Patent No. 4,683, 202), ligase chain reaction (Barany, 1991), self-sustained sequence replication (Guatelli et al., 1990), transcriptional amplification system (Kwoh et al., 1989), Q-Beta Replicase (Lizardi et al., 1988), rolling circle replication (U. S. Patent No. 5,854, 033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

In a further aspect, the present invention relates to a method for predicting lymph node metastasis in a patient afflicted with solid cancer after radiotherapy treatment, comprising the steps of: i) determining the expression level of VEGF-C in a biological sample obtained from said patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient will not have a lymph node metastasis when the level determined at step i) is lower than the predetermined reference value or concluding that the patient will have a lymph node metastasis when the level determined at step i) is higher than the predetermined reference value.

In a further aspect, the present invention relates to a method for predicting cancer relapse in a patient afflicted with solid cancer after radiotherapy treatment, comprising the steps of: i) determining the expression level of VEGF-C in a biological sample obtained from said patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient will not have a cancer relapse when the level determined at step i) is lower than the predetermined reference value or concluding that the patient will have a cancer relapse when the level determined at step i) is higher than the predetermined reference value.

In a further aspect, the present invention relates to a method for predicting the survival time of a patient afflicted with solid cancer after radiotherapy treatment, comprising the steps of: i) determining the expression level of VEGF-C in a biological sample obtained from the patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient will have a long survival time when the level determined at step i) is lower than the predetermined reference value or concluding that the patient will have a short survival time when the level determined at step i) is higher than the predetermined reference value.

The method of the present invention is particularly suitable for predicting the duration of the overall survival (OS), progression-free survival (PFS) and/or the disease-free survival (DFS) of the cancer patient. Those of skill in the art will recognize that OS survival time is generally based on and expressed as the percentage of people who survive a certain type of cancer for a specific amount of time. In general, OS rates do not specify whether cancer survivors are still undergoing treatment at five years or if they've become cancer-free (achieved remission). DFS gives more specific information and is the number of people with a particular cancer who achieve remission. Also, progression-free survival (PFS) rates (the number of people who still have cancer, but their disease does not progress) includes people who may have had some success with treatment, but the cancer has not disappeared completely. As used herein, the expression "short survival time" indicates that the patient will have a survival time that will be lower than the median (or mean) observed in the general population of patients suffering from said cancer. When the patient will have a short survival time, it is meant that the patient will have a "poor prognosis". Inversely, the expression "long survival time" indicates that the patient will have a survival time that will be higher than the median (or mean) observed in the general population of patients suffering from said cancer. When the patient will have a long survival time, it is meant that the patient will have a "good prognosis".

In some embodiment, the method of the invention is performed for predicting the overall survival (OS), progression-free survival (PFS) and/or the disease-free survival (DFS) of a patient afflicted with solid cancer receiving radiotherapy treatment.

5 A further aspect of the invention relates to a method of monitoring cancer progression in a patient afflicted with solid cancer after radiotherapy treatment by performing the method of the invention.

A further aspect of the invention relates to a method of monitoring radiotherapy treatment of a patient afflicted with solid cancer by performing the method of the invention.

10 In a further aspect, the present invention relates to a VEGF-C inhibitor for use in the treatment of solid cancer in a patient in need thereof wherein the patient was being classified as having a poor prognosis by the method as above described.

The solid cancer referred to in the methods and uses described herein above may be for example a head and neck squamous cell carcinoma (HNSCC) or a medulloblastoma (MDB).

15 As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patients at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a patient
20 having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a patient beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen
25 may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which
30 may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an

illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

The term “VEGF-C inhibitor” has its general meaning in the art and refers to a compound that selectively blocks or inactivates the VEGF-C. The term “VEGF-C inhibitor” also refers to a compound that selectively blocks the binding of VEGF-C to its receptors (such as VEGFR-3 and VEGFR-2). The term “VEGF-C inhibitor” also refers to a compound able to prevent the action of VEGF-C for example by inhibiting the VEGF-C controls of downstream effectors such as inhibiting the activation of the KRAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways. As used herein, the term “selectively blocks or inactivates” refers to a compound that preferentially binds to and blocks or inactivates VEGF-C with a greater affinity and potency, respectively, than its interaction with the other sub-types of the VEGF family. Compounds that block or inactivate VEGF-C, but that may also block or inactivate other VEGF sub-types, as partial or full inhibitors, are contemplated. The term “VEGF-C inhibitor” also refers to a compound that inhibits VEGF-C expression. Typically, a VEGF-C inhibitor is a small organic molecule, a polypeptide, an aptamer, an antibody, an oligonucleotide, a ribozyme or a CRISPR.

Tests and assays for determining whether a compound is a VEGF-C inhibitor are well known by the skilled person in the art such as described in WO2011/127519; WO2011/071577; WO0152875; WO2011127519.

In one embodiment of the invention, VEGF-C inhibitors include but are not limited to the anti-VEGF-C antibodies. Anti-VEGF-C antibodies are available and described in the art (such as in WO2011/127519; WO2011/071577; WO2011/127519).

An example of anti-VEGF-C antibody which can be used in the context of the invention is identified as “VGX-100” in WO2011/127519 and WO2011/071577.

Other examples of anti-VEGF-C antibodies are described in WO2011/071577, such as in particular those respectively identified as “VC4.5” and “VC1.12” which can, among others, be used in the context of the invention.

In some embodiments, the VEGF-C inhibitor is a VEGFR-3 antagonist (WO2016/184793). In some embodiments, the VEGF-C inhibitor is a VEGFR-2 antagonist (WO2016/184793).

In another embodiment, the VEGF-C inhibitor of the invention is an antibody (the term including "antibody portion") directed against VEGF-C, VEGFR-3 or VEGFR-2.

In one embodiment of the antibodies or portions thereof described herein, the antibody is a monoclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a polyclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a humanized antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a chimeric antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a light chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a heavy chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fab portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a F(ab')₂ portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fc portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fv portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a variable domain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises one or more CDR domains of the antibody.

As used herein, "antibody" includes both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and monovalent and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. The antibody may be a human or nonhuman antibody. A nonhuman antibody may be humanized by recombinant methods to reduce its immunogenicity in man.

Antibodies are prepared according to conventional methodology. Monoclonal antibodies may be generated using the method of Kohler and Milstein (Nature, 256:495, 1975). To prepare monoclonal antibodies useful in the invention, a mouse or other appropriate host animal is immunized at suitable intervals (e.g., twice-weekly, weekly, twice-monthly or monthly) with antigenic forms of VEGF-C. The animal may be administered a final "boost" of antigen within one week of sacrifice. It is often desirable to use an immunologic adjuvant during immunization. Suitable immunologic adjuvants include Freund's complete adjuvant, Freund's incomplete adjuvant, alum, Ribi adjuvant, Hunter's Titermax, saponin adjuvants such

as QS21 or Quil A, or CpG-containing immunostimulatory oligonucleotides. Other suitable adjuvants are well-known in the field. The animals may be immunized by subcutaneous, intraperitoneal, intramuscular, intravenous, intranasal or other routes. A given animal may be immunized with multiple forms of the antigen by multiple routes.

5 Briefly, the antigen may be provided as synthetic peptides corresponding to antigenic regions of interest in VEGF-C. Following the immunization regimen, lymphocytes are isolated from the spleen, lymph node or other organ of the animal and fused with a suitable myeloma cell line using an agent such as polyethylene glycol to form a hydridoma. Following fusion, cells are placed in media permissive for growth of hybridomas but not the fusion
10 partners using standard methods, as described (Coding, Monoclonal Antibodies: Principles and Practice: Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology, 3rd edition, Academic Press, New York, 1996). Following culture of the hybridomas, cell supernatants are analyzed for the presence of antibodies of the desired specificity, i.e., that selectively bind the antigen. Suitable analytical techniques
15 include ELISA, flow cytometry, immunoprecipitation, and western blotting. Other screening techniques are well-known in the field. Preferred techniques are those that confirm binding of antibodies to conformationally intact, natively folded antigen, such as non-denaturing ELISA, flow cytometry, and immunoprecipitation.

Significantly, as is well-known in the art, only a small portion of an antibody
20 molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The Fc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has
25 been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated as Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a
30 covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDRS). The CDRs, and in particular the CDRS regions, and more particularly the heavy chain CDRS, are largely responsible for antibody specificity.

It is now well-established in the art that the non CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody.

This invention provides in certain embodiments compositions and methods that include humanized forms of antibodies. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby incorporated by reference. The above U.S. Pat. Nos. 5,585,089 and 5,693,761, and WO 90/07861 also propose four possible criteria which may be used in designing the humanized antibodies. The first proposal was that for an acceptor to use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or to use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid residue at the framework positions at which the amino acid is predicted to have a side chain atom within 3Å of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make

humanized antibodies. One of ordinary skill in the art will be familiar with other methods for antibody humanization.

In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgG1, IgG2, IgG3, IgG4, IgA and IgM molecules. A "humanized" antibody retains a similar antigenic specificity as the original antibody. However, using certain methods of humanization, the affinity and/or specificity of binding of the antibody may be increased using methods of "directed evolution", as described by Wu et al., *J. Mol. Biol.* 294:151, 1999, the contents of which are incorporated herein by reference.

Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of which are incorporated herein by reference. These animals have been genetically modified such that there is a functional deletion in the production of endogenous (e.g., murine) antibodies. The animals are further modified to contain all or a portion of the human germ-line immunoglobulin gene locus such that immunization of these animals will result in the production of fully human antibodies to the antigen of interest. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (KAMA) responses when administered to humans.

In vitro methods also exist for producing human antibodies. These include phage display technology (U.S. Pat. Nos. 5,565,332 and 5,573,905) and *in vitro* stimulation of human B cells (U.S. Pat. Nos. 5,229,275 and 5,567,610). The contents of these patents are incorporated herein by reference.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂ Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which

the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

The various antibody molecules and fragments may derive from any of the commonly known immunoglobulin classes, including but not limited to IgA, secretory IgA, IgE, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgG1, IgG2, IgG3 and IgG4. In a preferred embodiment, the VEGF-C inhibitor of the invention is a Human IgG4.

In some embodiments, the invention provides a multi-specific antibody comprising a first antigen binding site from an antibody of the present invention directed against VEGF-C, VEGFR-3 or VEGFR-2 and at least one second antigen binding site.

In some embodiments, the second antigen-binding site is used for recruiting a killing mechanism such as, for example, by binding an antigen on a human effector cell as a BiTE (Bispecific T-Cell engager) antibody which is a bispecific scFv2 directed against target antigen and CD3 on T cells described in US7235641, or by binding a cytotoxic agent or a second therapeutic agent. As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, for instance lymphocytes (such as B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, mast cells and granulocytes, such as neutrophils, eosinophils and basophils. Some effector cells express specific Fc receptors (FcRs) and carry out specific immune functions. In some embodiments, an effector cell is capable of inducing ADCC, such as a natural killer cell. For example, monocytes, macrophages, which express FcRs, are involved in specific killing of target cells and presenting antigens to other components of the immune system. In some embodiments, an effector cell may phagocytose a target antigen or target cell. The expression of a particular FcR on an effector cell may be regulated by humoral factors such as cytokines. An effector cell can phagocytose a target antigen or phagocytose or lyse a target cell. Suitable cytotoxic agents and second therapeutic agents are exemplified below, and include toxins (such as radiolabeled peptides), chemotherapeutic agents and prodrugs

In some embodiments, the second antigen-binding site binds a tissue-specific antigen, promoting localization of the bispecific antibody to a specific tissue.

In some embodiments, the second antigen-binding site binds to an antigen located on the same type of cell as the [VEGF-C, VEGFR-3 or VEGFR-2]-expressing cell, typically a tumor-associated antigen (TAA), but has a binding specificity different from that of the first antigen-binding site. Such multi- or bispecific antibodies can enhance the specificity of the tumor cell binding and/or engage multiple effector pathways. Exemplary TAAs include carcinoembryonic antigen (CEA), prostate specific antigen (PSA), RAGE (renal antigen), α -fetoprotein, CAMEL (CTL-recognized antigen on melanoma), CT antigens (such as MAGE-B5, -B6, -C2, -C3, and D; Mage-12; CT10; NY-ESO-1, SSX-2, GAGE, BAGE, MAGE, and SAGE), mucin antigens (e.g., MUC1, mucin-CA125, etc.), ganglioside antigens, tyrosinase, gp75, c-Met, Marti, MelanA, MUM-1, MUM-2, MUM-3, HLA-B7, Ep-CAM or a cancer-associated integrin, such as $\alpha 5 \beta 3$ integrin. Alternatively, the second antigen-binding site binds to a different epitope of [VEGF-C, VEGFR-3 or VEGFR-2]. The second antigen-binding site may alternatively bind an angiogenic factor or other cancer-associated growth factor, such as a vascular endothelial growth factor, a fibroblast growth factor, epidermal growth factor, angiogenin or a receptor of any of these, particularly receptors associated with cancer progression.

In some embodiments, the second antigen-binding site is from a second antibody or ADCC of the invention, such as the antibody of the present invention.

Exemplary formats for the multispecific antibody molecules of the invention include, but are not limited to (i) two antibodies cross-linked by chemical heteroconjugation, one with a specificity to [VEGF-C, VEGFR-3 or VEGFR-2] and another with a specificity to a second antigen; (ii) a single antibody that comprises two different antigen-binding regions; (iii) a single-chain antibody that comprises two different antigen-binding regions, e.g., two scFvs linked in tandem by an extra peptide linker; (iv) a dual-variable-domain antibody (DVD-Ig), where each light chain and heavy chain contains two variable domains in tandem through a short peptide linkage (Wu et al., Generation and Characterization of a Dual Variable Domain Immunoglobulin (DVD-IgTM) Molecule, In : Antibody Engineering, Springer Berlin Heidelberg (2010)); (v) a chemically-linked bispecific (Fab')₂ fragment; (vi) a Tandab, which is a fusion of two single chain diabodies resulting in a tetravalent bispecific antibody that has two binding sites for each of the target antigens; (vii) a flexibody, which is a combination of scFvs with a diabody resulting in a multivalent molecule; (viii) a so called "dock and lock" molecule, based on the "dimerization and docking domain" in Protein Kinase A, which, when

applied to Fabs, can yield a trivalent bispecific binding protein consisting of two identical Fab fragments linked to a different Fab fragment; (ix) a so-called Scorpion molecule, comprising, e.g., two scFvs fused to both termini of a human Fab-arm; and (x) a diabody. Another exemplary format for bispecific antibodies is IgG-like molecules with complementary CH3 domains to force heterodimerization. Such molecules can be prepared using known technologies, such as, e.g., those known as Triomab/Quadroma (Trion Pharma/Fresenius Biotech), Knob-into-Hole (Genentech), CrossMAb (Roche) and electrostatically-matched (Amgen), LUZ-Y (Genentech), Strand Exchange Engineered Domain body (SEEDbody)(EMD Serono), Biclonic (Merus) and DuoBody (Genmab A/S) technologies.

In some embodiments, the bispecific antibody is obtained or obtainable via a controlled Fab-arm exchange, typically using DuoBody technology. *In vitro* methods for producing bispecific antibodies by controlled Fab-arm exchange have been described in WO2008119353 and WO 2011131746 (both by Genmab A/S). In one exemplary method, described in WO 2008119353, a bispecific antibody is formed by "Fab-arm" or "half-molecule" exchange (swapping of a heavy chain and attached light chain) between two monospecific antibodies, both comprising IgG4-like CH3 regions, upon incubation under reducing conditions. The resulting product is a bispecific antibody having two Fab arms which may comprise different sequences. In another exemplary method, described in WO2011131746, bispecific antibodies of the present invention are prepared by a method comprising the following steps, wherein at least one of the first and second antibodies is the antibody of the present invention : a) providing a first antibody comprising an Fc region of an immunoglobulin, said Fc region comprising a first CH3 region; b) providing a second antibody comprising an Fc region of an immunoglobulin, said Fc region comprising a second CH3 region; wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions; c) incubating said first antibody together with said second antibody under reducing conditions; and d) obtaining said bispecific antibody, wherein the first antibody is the antibody of the present invention and the second antibody has a different binding specificity, or vice versa. The reducing conditions may, for example, be provided by adding a reducing agent, e.g. selected from 2-mercaptoethylamine, dithiothreitol and tris(2-carboxyethyl)phosphine. Step d) may further comprise restoring the conditions to become non-reducing or less reducing, for example by removal of a reducing agent, e.g. by desalting. Preferably, the sequences of the first and second CH3 regions are different, comprising only a few, fairly conservative,

asymmetrical mutations, such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions. More details on these interactions and how they can be achieved are provided in WO2011131746, which is hereby incorporated by reference in its entirety. The following are
5 exemplary embodiments of combinations of such asymmetrical mutations, optionally wherein one or both Fc-regions are of the IgG1 isotype.

In another embodiment, the antibody according to the invention is a single domain antibody. The term "single domain antibody" (sdAb) or "VHH" refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which
10 are naturally devoid of light chains. Such VHH are also called "nanobody®". According to the invention, sdAb can particularly be llama sdAb. The term "VHH" refers to the single heavy chain having 3 complementarity determining regions (CDRs): CDR1, CDR2 and CDR3. The term "complementarity determining region" or "CDR" refers to the hypervariable amino acid sequences which define the binding affinity and specificity of the VHH.

15 The VHH according to the invention can readily be prepared by an ordinarily skilled artisan using routine experimentation. The VHH variants and modified form thereof may be produced under any known technique in the art such as in-vitro maturation.

VHHs or sdAbs are usually generated by PCR cloning of the V-domain repertoire from blood, lymph node, or spleen cDNA obtained from immunized animals into a phage
20 display vector, such as pHEN2. Antigen-specific VHHs are commonly selected by panning phage libraries on immobilized antigen, e.g., antigen coated onto the plastic surface of a test tube, biotinylated antigens immobilized on streptavidin beads, or membrane proteins expressed on the surface of cells. However, such VHHs often show lower affinities for their antigen than VHHs derived from animals that have received several immunizations. The high
25 affinity of VHHs from immune libraries is attributed to the natural selection of variant VHHs during clonal expansion of B-cells in the lymphoid organs of immunized animals. The affinity of VHHs from non-immune libraries can often be improved by mimicking this strategy *in vitro*, i.e., by site directed mutagenesis of the CDR regions and further rounds of panning on immobilized antigen under conditions of increased stringency (higher temperature, high or
30 low salt concentration, high or low pH, and low antigen concentrations). VHHs derived from camelid are readily expressed in and purified from the E. coli periplasm at much higher levels than the corresponding domains of conventional antibodies. VHHs generally display high solubility and stability and can also be readily produced in yeast, plant, and mammalian cells. For example, the "Hamers patents" describe methods and techniques for generating VHH

against any desired target (see for example US 5,800,988; US 5,874, 541 and US 6,015,695). The “Hamers patents” more particularly describe production of VHHs in bacterial hosts such as *E. coli* (see for example US 6,765,087) and in lower eukaryotic hosts such as moulds (for example *Aspergillus* or *Trichoderma*) or in yeast (for example *Saccharomyces*,
5 *Kluyveromyces*, *Hansenula* or *Pichia*) (see for example US 6,838,254).

In one embodiment, the VEGF-C inhibitor of the invention is a VEGF-C expression inhibitor, VEGFR-3 and/or VEGFR-2 expression inhibitor.

The term “expression” when used in the context of expression of a gene or nucleic acid refers to the conversion of the information, contained in a gene, into a gene product. A
10 gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include messenger RNAs, which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins (e.g., VEGF-C, VEGFR-3 and VEGFR-2) modified by, for example, methylation, acetylation,
15 phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, myristilation, and glycosylation.

An “inhibitor of expression” refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene.

VEGF-C, VEGFR-3 and VEGFR-2 expression inhibitors for use in the present
20 invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of VEGF-C, VEGFR-3 and VEGFR-2 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of VEGF-C, VEGFR-3 and VEGFR-2 proteins, and thus activity, in a cell. For example,
25 antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding VEGF-C, VEGFR-3 and VEGFR-2 can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically alleviating gene expression of genes whose sequence is known are well known in the art (e.g.
30 see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as expression inhibitors for use in the present invention. Gene expression can be reduced by contacting the subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a

small double stranded RNA, such that VEGF-C, VEGFR-3 and VEGFR-2 expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002);
5 McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO01/36646, WO99/32619, and WO01/68836).

Ribozymes can also function as expression inhibitors for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of
10 RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are
15 initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The
20 suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as VEGF-C, VEGFR-3 and VEGFR-2 inhibitors can be prepared by known methods. These include techniques for
25 chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by *in vitro* or *in vivo* transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the
30 invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered *in vivo* alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells and preferably cells expressing VEGF-C, VEGFR-3 and VEGFR-2.

5 Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences.

10 Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a

15 retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA.

20 Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the

25 steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana

30 Press, Inc., Clifton, N.J., 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages

such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells *in vivo*. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In one embodiment of the invention, VEGF-C, VEGFR-3 and VEGFR-2 expression inhibitors include but are not limited to siRNAs and shRNA such as described in Liu et al., 2015 and Ye et al., 2015.

Typically the inhibitors according to the invention as described above are administered to the patient in a therapeutically effective amount.

By a "therapeutically effective amount" of the inhibitor of the present invention as above described is meant a sufficient amount of the inhibitor for treating solid cancer at a

reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the inhibitors and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific inhibitor employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific inhibitor employed; the duration of the treatment; drugs used in combination or coincidental with the specific inhibitor employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the inhibitor at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the inhibitor of the present invention for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the inhibitor of the present invention, preferably from 1 mg to about 100 mg of the inhibitor of the present invention. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

In a particular embodiment, the compound according to the invention may be used in a concentration between 0.01 μ M and 20 μ M, particularly, the compound of the invention may be used in a concentration of 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 μ M.

In a further aspect, the present invention relates to the compound according to the invention for use in the treatment of solid cancer in a patient in need thereof in combination with radiotherapy treatment wherein the patient was being classified as having a poor prognosis by the method as above described.

In some embodiments, the VEGF-C inhibitor of the invention is administered sequentially or concomitantly with the radiotherapy treatment, herein defined as a photon (X) radiotherapy or as a proton (P) radiotherapy.

In a further aspect, the present invention relates to the compound according to the invention in combination with one or more anti-cancer compound for use in the treatment of solid cancer in a patient in need thereof wherein the patient was being classified as having a poor prognosis by the method as above described.

In a further aspect, the present invention relates to the compound according to the invention in combination with one or more anti-angiogenic compound or anti-lymphangiogenic compound for use in the treatment of solid cancer in a patient in need thereof wherein the patient was being classified as having a poor prognosis by the method as above described.

The term “anti-cancer compound” has its general meaning in the art and refers to compounds used in anti-cancer therapy such as anti-angiogenic compound, tyrosine kinase inhibitors, tyrosine kinase receptor (TKR) inhibitors, Vascular Endothelial Growth Factor Receptors (VEGFRs) pathway inhibitors, interferon therapy, anti-HER2 compounds, anti-EGFR compounds, alkylating agents, anti-metabolites, immunotherapeutic agents, Interferons (IFNs), Interleukins, and chemotherapeutic agents such as described below.

The term “anti-angiogenic compound” has its general meaning in the art and refers to compounds used in anti-angiogenic therapy such as tyrosine kinase inhibitors, anti-angiogenic tyrosine kinase receptor (TKR) inhibitors, anti-angiogenics targeting the Vascular Endothelial Growth Factor Receptors (VEGFRs) pathway, interferon therapy and anti-HER2 compounds such as Trastuzumab (herceptin) and pertuzumab. In one embodiment, the term “anti-angiogenic compound” refers to Sunitinib (Sutent), an anti-angiogenic TKR inhibitor of VEGFRs, platelet-derived growth factor receptors (PDGF-Rs), and c-kit.

The term “tyrosine kinase inhibitor” refers to any of a variety of therapeutic agents or drugs that act as selective or non-selective inhibitors of receptor and/or non-receptor tyrosine kinases. Tyrosine kinase inhibitors and related compounds are well known in the art and described in U.S Patent Publication 2007/0254295, which is incorporated by reference herein in its entirety. It will be appreciated by one of skill in the art that a compound related to a tyrosine kinase inhibitor will recapitulate the effect of the tyrosine kinase inhibitor, e.g., the related compound will act on a different member of the tyrosine kinase signaling pathway to produce the same effect as would a tyrosine kinase inhibitor of that tyrosine kinase. Examples of tyrosine kinase inhibitors and related compounds suitable for use in methods of embodiments of the present invention include, but are not limited to, sunitinib (Sutent; SU11248), Axitinib, pazopanib (Votrient), cabozantinib, dasatinib (BMS-354825), PP2, BEZ235, saracatinib, gefitinib (Iressa), erlotinib (Tarceva; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec; STI571), leflunomide (SU101), vandetanib (Zactima; ZD6474), MK-2206 (8-[4-aminocyclobutyl]phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one hydrochloride) derivatives thereof, analogs thereof, and

combinations thereof. Additional tyrosine kinase inhibitors and related compounds suitable for use in the present invention are described in, for example, U.S. Patent Publication 2007/0254295, U.S. Pat. Nos. 5,618,829, 5,639,757, 5,728,868, 5,804,396, 6,100,254, 6,127,374, 6,245,759, 6,306,874, 6,313,138, 6,316,444, 6,329,380, 6,344,459, 6,420,382, 6,479,512, 6,498,165, 6,544,988, 6,562,818, 6,586,423, 6,586,424, 6,740,665, 6,794,393, 6,875,767, 6,927,293, and 6,958,340, all of which are incorporated by reference herein in their entirety. In certain embodiments, the tyrosine kinase inhibitor is a small molecule kinase inhibitor that has been orally administered to the patient of at least one Phase I clinical trial, more preferably at least one Phase II clinical, even more preferably at least one Phase III clinical trial, and most preferably approved by the FDA for at least one hematological or oncological indication. Examples of such inhibitors include, but are not limited to, Gefitinib, Erlotinib, Lapatinib, Canertinib, BMS-599626 (AC-480), Neratinib, KRN-633, CEP-11981, Imatinib, Nilotinib, Dasatinib, AZM-475271, CP-724714, TAK-165, Sunitinib, Vatalanib, CP-547632, Vandetanib, Bosutinib, Lestaurtinib, Tandutinib, Midostaurin, Enzastaurin, AEE-788, Pazopanib, Axitinib, Motasenib, OSI-930, Cediranib, KRN-951, Dovitinib, Seliciclib, SNS-032, PD-0332991, MKC-I (Ro-317453; R-440), Sorafenib, ABT-869, Brivanib (BMS-582664), SU-14813, Telatinib, SU-6668, (TSU-68), L-21649, MLN-8054, AEW-541, and PD-0325901.

In one embodiment, the term "anti-angiogenic compound" refers to compounds targeting the vascular endothelial growth factor (VEGF) pathway such as anti-VEGF antibody bevacizumab (Avastin) and VEGF receptor tyrosine kinase inhibitor (TKI) compounds such as sunitinib (Sutent), vandetanib (Zactima), pazopanib (Votrient), sorafenib (Nexavar) and cediranib.

According to the present invention, the compound of the invention is administered sequentially or concomitantly with one or more anti-cancer compound and/or anti-angiogenic compound.

According to the invention, the compound of the present invention is administered to the patient in the form of a pharmaceutical composition. Typically, the compound of the present invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. "Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable

carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

Typically, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must 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. Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The compound of the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and

such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. 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. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

10 Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by

15 incorporating the various sterilized agent of the present inventions into a sterile vehicle which contains the 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 typical methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the compound of the present invention plus any additional desired

20 ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is

25 therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are

30 especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. Some variation in dosage will necessarily occur depending on the condition of the patient being treated. The person

responsible for administration will, in any event, determine the appropriate dose for the individual patient.

In some embodiments, the compound of the present invention is administered sequentially or concomitantly with one or more therapeutic active agent such as chemotherapeutic.

In some embodiments, the compound of the present invention is administered with a chemotherapeutic agent. The term "chemotherapeutic agent" refers to chemical compounds that are effective in inhibiting tumor growth. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin (11 and calicheamicin 211, see, e.g., Agnew Chem Intl. Ed. Engl. 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromomorphores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, canninomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptomycin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as

ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophospharnide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofiran; spirogennanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylarnine; trichothecenes (especially T-2 toxin, verracurin A, roridinA and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobromtol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are antihormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In some embodiments, the compound of the present invention is administered with a targeted cancer therapy. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules ("molecular targets") that are involved in the growth, progression, and spread of cancer. Targeted cancer therapies are sometimes called "molecularly targeted drugs", "molecularly targeted therapies", "precision medicines", or similar names. In some embodiments, the targeted therapy consists of administering the patient with a tyrosine kinase inhibitor as defined above.

In some embodiments, compound of the present invention is administered with an immunotherapeutic agent. The term "immunotherapeutic agent," as used herein, refers to a compound, composition or treatment that indirectly or directly enhances, stimulates or increases the body's immune response against cancer cells and/or that decreases the side effects of other anticancer therapies. Immunotherapy is thus a therapy that directly or indirectly stimulates or enhances the immune system's responses to cancer cells and/or lessens the side effects that may have been caused by other anti-cancer agents. Immunotherapy is also referred to in the art as immunologic therapy, biological therapy biological response modifier therapy and biotherapy. Examples of common immunotherapeutic agents known in the art include, but are not limited to, cytokines, cancer vaccines, monoclonal antibodies and non-cytokine adjuvants. Alternatively the immunotherapeutic treatment may consist of administering the patient with an amount of immune cells (T cells, NK cells, dendritic cells, B cells...). Immunotherapeutic agents can be non-specific, i.e. boost the immune system generally so that the human body becomes more effective in fighting the growth and/or spread of cancer cells, or they can be specific, i.e. targeted to the cancer cells themselves. Immunotherapy regimens may combine the use of non-specific and specific immunotherapeutic agents. Non-specific immunotherapeutic agents are substances that stimulate or indirectly improve the immune system. Non-specific immunotherapeutic agents have been used alone as a main therapy for the treatment of cancer, as well as in addition to a main therapy, in which case the non-specific immunotherapeutic agent functions as an adjuvant to enhance the effectiveness of other therapies (e.g. cancer vaccines). Non-specific immunotherapeutic agents can also function in this latter context to reduce the side effects of other therapies, for example, bone marrow suppression induced by certain chemotherapeutic agents. Non-specific immunotherapeutic agents can act on key immune system cells and cause secondary responses, such as increased production of cytokines and immunoglobulins. Alternatively, the agents can themselves comprise cytokines. Non-specific immunotherapeutic agents are generally classified as cytokines or non-cytokine adjuvants. A number of cytokines have found application in the treatment of cancer either as general non-specific immunotherapies designed to boost the immune system, or as adjuvants provided with other therapies. Suitable cytokines include, but are not limited to, interferons, interleukins and colony-stimulating factors. Interferons (IFNs) contemplated by the present invention include the common types of IFNs, IFN-alpha (IFN- α), IFN-beta (IFN- β) and IFN-gamma (IFN- γ). IFNs can act directly on cancer cells, for example, by slowing their growth, promoting their development into cells with more normal behaviour and/or increasing their

production of antigens thus making the cancer cells easier for the immune system to recognise and destroy. IFNs can also act indirectly on cancer cells, for example, by slowing down angiogenesis, boosting the immune system and/or stimulating natural killer (NK) cells, T cells and macrophages. Recombinant IFN-alpha is available commercially as Roferon (Roche Pharmaceuticals) and Intron A (Schering Corporation). Interleukins contemplated by the present invention include IL-2, IL-4, IL-11 and IL-12. Examples of commercially available recombinant interleukins include Proleukin® (IL-2; Chiron Corporation) and Neumega® (IL-12; Wyeth Pharmaceuticals). Zymogenetics, Inc. (Seattle, Wash.) is currently testing a recombinant form of IL-21, which is also contemplated for use in the combinations of the present invention. Colony-stimulating factors (CSFs) contemplated by the present invention include granulocyte colony stimulating factor (G-CSF or filgrastim), granulocyte-macrophage colony stimulating factor (GM-CSF or sargramostim) and erythropoietin (epoetin alfa, darbepoietin). Treatment with one or more growth factors can help to stimulate the generation of new blood cells in patients undergoing traditional chemotherapy. Accordingly, treatment with CSFs can be helpful in decreasing the side effects associated with chemotherapy and can allow for higher doses of chemotherapeutic agents to be used. Various-recombinant colony stimulating factors are available commercially, for example, Neupogen® (G-CSF; Amgen), Neulasta (pelfilgrastim; Amgen), Leukine (GM-CSF; Berlex), Procrit (erythropoietin; Ortho Biotech), Epogen (erythropoietin; Amgen), Arnesp (erythropoietin). In addition to having specific or non-specific targets, immunotherapeutic agents can be active, i.e. stimulate the body's own immune response, or they can be passive, i.e. comprise immune system components that were generated external to the body. Passive specific immunotherapy typically involves the use of one or more monoclonal antibodies that are specific for a particular antigen found on the surface of a cancer cell or that are specific for a particular cell growth factor. Monoclonal antibodies may be used in the treatment of cancer in a number of ways, for example, to enhance a patient's immune response to a specific type of cancer, to interfere with the growth of cancer cells by targeting specific cell growth factors, such as those involved in angiogenesis, or by enhancing the delivery of other anticancer agents to cancer cells when linked or conjugated to agents such as chemotherapeutic agents, radioactive particles or toxins. Monoclonal antibodies currently used as cancer immunotherapeutic agents that are suitable for inclusion in the combinations of the present invention include, but are not limited to, rituximab (Rituxan®), trastuzumab (Herceptin®), ibritumomab tiuxetan (Zevalin®), tositumomab (Bexxar®), cetuximab (C-225, Erbitux®), bevacizumab (Avastin®), gemtuzumab ozogamicin (Mylotarg®), alemtuzumab (Campath®), and BL22.

Other examples include anti-CTLA4 antibodies (e.g. Ipilimumab), anti-PD1 antibodies (nivolumab, pembrolizumab), anti-PDL1 antibodies (atezolizumab), anti-TIMP3 antibodies, anti-LAG3 antibodies, anti-B7H3 antibodies, anti-B7H4 antibodies or anti-B7H6 antibodies.

In some embodiments, antibodies include B cell depleting antibodies. Typical B cell depleting antibodies include but are not limited to anti-CD20 monoclonal antibodies [e.g. Rituximab (Roche), Ibritumomab tiuxetan (Bayer Schering), Tositumomab (GlaxoSmithKline), AME-133v (Applied Molecular Evolution), Ocrelizumab (Roche), Ofatumumab (HuMax-CD20, Gemnab), TRU-015 (Trubion) and IMMU-106 (Immunomedics)], an anti-CD22 antibody [e.g. Epratuzumab, Leonard et al., Clinical Cancer Research (2004) 10: 5327-5334], anti-CD79a antibodies, anti-CD27 antibodies, or anti-CD19 antibodies (e.g. U.S. Pat. No. 7,109,304), anti-BAFF-R antibodies (e.g. Belimumab, GlaxoSmithKline), anti-APRIL antibodies (e.g. anti-human APRIL antibody, ProSci inc.), and anti-IL-6 antibodies [e.g. previously described by De Benedetti et al., J Immunol (2001) 166: 4334-4340 and by Suzuki et al., Europ J of Immunol (1992) 22 (8) 1989-1993, fully incorporated herein by reference].

The immunotherapeutic treatment may consist of allografting, in particular, allograft with hematopoietic stem cell HSC. The immunotherapeutic treatment may also consist in an adoptive immunotherapy as described by Nicholas P. Restifo, Mark E. Dudley and Steven A. Rosenberg "Adoptive immunotherapy for cancer: harnessing the T cell response, Nature Reviews Immunology, Volume 12, April 2012). In adoptive immunotherapy, the patient's circulating lymphocytes, NK cells, are isolated amplified *in vitro* and readministered to the patient. The activated lymphocytes or NK cells are most preferably the patient's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*.

In one embodiment, said additional active compounds may be contained in the same composition or administrated separately.

In another embodiment, the pharmaceutical composition of the invention relates to combined preparation for simultaneous, separate or sequential use in the treatment of solid cancer in a patient in need thereof wherein the patient was being classified as having a poor prognosis by the method as above described.

In a further aspect, the present invention also relates to a method for treating solid cancer in a patient in need thereof wherein the patient was being classified as having a poor prognosis by the method as above described, comprising the step of administering to said patient the compound of the invention.

The solid cancer referred to in the methods and uses described herein above may be for example a head and neck squamous cell carcinoma (HNSCC) or a medulloblastoma (MDB).

5 The invention also provides kits comprising the compound of the invention. Kits containing the compound of the invention find use in therapeutic methods.

In a further aspect, the present invention relates to a method of screening a candidate compound for use as a drug for the treatment of solid cancer in a patient in need thereof wherein the patient was being classified as having a poor prognosis by the method as above described, wherein the method comprises the steps of:

- 10 - providing a VEGF-C and VEGF-C receptor (VEGFR-3 and VEGFR-2),
providing a cell, tissue sample or organism expressing a VEGF-C and VEGF-C receptor (VEGFR-3 and VEGFR-2),
- providing radiotherapy treatment,
 - providing a candidate compound such as small organic molecule, an
- 15 oligonucleotide, a polypeptide, an aptamer, antibody or an intra-antibody,
- measuring the lymphatic vessel density and lymph node metastasis,
 - selecting positively candidate compounds that inhibit lymph node metastasis induced by the radiotherapy treatment.

Methods for measuring lymph node metastasis are well known in the art. For example,

20 the lymph node metastasis is measured such as described in the example. For example, measuring the lymphatic vessel density and lymph node metastasis involves determining a Ki on the VEGF-C receptor cloned and transfected in a stable manner into a CHO cell line, measuring the VEGF-C downstream signalling, measuring lymphatic vessel density, measuring inhibition of VEGF-C induced endothelial cell proliferation, measuring VEGFR-3

25 and VEGFR-2 phosphorylation, and measuring KRAS/RAF/MEK/ERK and PI3K/AKT/mTOR signaling.

In a further aspect, the present invention relates to a method for treating solid cancer, for example a head and neck squamous cell carcinoma (HNSCC) or a medulloblastoma (MDB), in a patient in need thereof, comprising the step of administering to said patient a

30 VEGF-C inhibitor in combination with radiotherapy treatment (herein defined as a photon (X) radiotherapy or a proton (P) radiotherapy), for example in combination with proton radiotherapy.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. CAL33 proliferative ability following multiple X or P irradiations

Counts of CAL33 cells following multiple low (2 Gy) or high (8 Gy) dose(s) of P or X irradiation and cell expansion after the third irradiation (CR-MI). The values correspond to fold increase, as compared to the viable cell number at 24h after cell seeding. Significantly decreased viable cell counts, as compared to CT: #, $p < 0.05$; ###, $p < 0.001$. Significantly increased viable cell counts for comparisons between X and P groups: *, $p < 0.05$; **, $p < 0.01$. CT, control (non-irradiated cells).

Figure 2. VEGF-C protein expression levels and regulation in CAL33 cells following P or X irradiation. (A) VEGF-C protein levels at 48h post-single irradiation (AR-SI): * and *, significantly ($p < 0.05$) increased levels after a low (2 Gy) or high (8 Gy) dose of P and X irradiation, respectively, as compared to CT; #, significantly decreased levels after a high dose of P, as compared to X irradiation; §, significantly increased levels after a high, as compared to a low X irradiation dose; (B) VEGF-C protein levels after cell expansion following the third irradiation (CR-MI): * and *, significantly increased levels after low and high doses of P and X irradiation, respectively, as compared to CT; Concentration in ng/ml, normalized to 1×10^6 cells, and represented as percentage of CT. #, significantly decreased levels after high doses of P, as compared to X irradiation; §, significantly increased levels after high, as compared to low doses of X irradiation; (C) Activity of a short vegf-c promoter (CR-MI); (D) Activity of an artificial promoter having three binding sites for NF- κ b (CR-MI); (E) Activity of a VEGF-C 3'UTR reporter gene (CR-MI). * and *, significantly ($p < 0.05$) increased promoter activity after P and X irradiation, respectively, as compared to CT; # and #, significantly decreased activity of MUT, as compared to WT vegf-c promoter after P and X irradiation, respectively; §, significantly decreased promoter activity after P, as compared to X irradiation; CT, control (non-irradiated cells); MUT, mutated, WT, wild type.

Figure 3. Evaluation of tumors generated following xenografting of either non-irradiated, P or X irradiated CAL33 cells in immunodeficient mice. (A) Average tumor volume (mm³); (B) Representative images of tumor xenografts; (C) Heatmap of ten most up- and down-regulated mouse genes in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors; (D) Heatmap of ten most up- and down-regulated human genes in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors; (E) Venn

diagrams showing common up-regulated and down-regulated human genes between P and X tumors. Framed genes are commonly expressed in P and X tumors. Selection is adjusted p value < 0.05 and logFC > 1.

Figure 4. Histology, immunofluorescence and quantitative gene expression of vascular and lymphatic markers in murine xenografts. (A) Representative images of HES staining, indicating increased necrosis (black arrowhead, delimited by dashed black lines) in CT and increased blood vessels density (white arrowhead showing collagen surrounding the vessels) in the irradiated cells-derived tumors; (B) Representative images of CD31 (endothelial cells, green) / α SMA (pericytes, red) / Hoechst (nuclei, blue) staining, showing anarchic blood vessels structures and lack of pericyte coverage of blood vessels in the irradiated cells-derived tumors; (C) Representative images of LYVE1 (lymphatic endothelial cells, red) / Hoechst (nuclei, blue) staining, showing different patterns of lymphatic vessels development in X (both periphery and interior of the tumor), P and CT (periphery of the tumor) groups; dashed white lines delimit the tumor edge; CT, control (tumors generated by non-irradiated cells); (D) Murine LYVE1, PDPN and PROX1 mRNA quantitative mRNA expression, as percentage of control (0 Gy).

Figure 5. Evaluation of vascular and lymphatic markers in biopsies from patients diagnosed with HNSCC. Representative images of immunohistochemistry for (A) PDPN and (B) CD31 expression: (1) oral and (2) hypopharyngeal localization; Left panels (1.a, 2.a) – primary tumor; Right panels (1.b, 2.b) – relapsed tumor in the same patient after surgery and chemo-X radiotherapy (brown, PDPN/CD31; blue, hematoxylin - nuclei); (C) quantitative PDPN, VEGF-C, LYVE1 and PROX1 mRNA expression, as percentage of control (0 Gy); * and **, significantly increased values ($p < 0.05$ and $p < 0.01$, respectively) post-, as compared to pre-X irradiation.

Figure 6. Clonogenic survival of CAL33 cells after irradiation with escalating doses of either P or X, indicating a Relative Biological Effectiveness of 1.1 in favor of P.

Figure 7. (A) Proliferation curves, (B) VEGF-C protein expression levels, activity of a (C) VEGF-C promoter and (D) artificial promotor having three NF- κ B binding sites, in CAL27 cells following high doses of either P or X irradiation (CR-MI setting). (A) #, ($p < 0.05$) and ## ($p < 0.01$) significantly decreased cell counts at 72h and 96h, respectively, post-irradiation with high doses of P or X, as compared to CT; (B) * and *, significantly increased VEGF-C protein levels after high doses of P and X irradiation, respectively, as compared to CT; #, significantly decreased levels after high doses of P, as compared to X irradiation; (C) #, significantly decreased activity in CT, P and X irradiated

cells transfected with a VEGF-C promotor with a MUT NF- κ B binding site, as compared to CT cells transfected with a VEGF-C promotor having a WT NF- κ B binding site; §, significantly decreased activity in cells transfected with a VEGF-C promotor having a MUT NF- κ B binding site, as compared to the corresponding cells transfected with a VEGF-C promoter having a WT NF- κ B binding site; **(D)** Lack of stimulation of NF- κ B promoter activity in irradiated cells; #, significantly decreased promoter activity in X irradiated, as compared to CT cells; CT, control (non-irradiated cells).

Figure 8. Heatmap of ten most up- and down-regulated mouse genes involved in angiogenesis, inflammation, metastasis, M1/M2 macrophage transition and proliferation in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors. Framed genes are commonly expressed in P and X tumors. Selection is $Abs(logFC) > 1$.

Figure 9. Heatmap of ten most up- and down-regulated human genes involved in angiogenesis, inflammation, metastasis, M1/M2 macrophage transition and proliferation in tumors generated by non-irradiated cells vs P or X tumors, and in P vs X tumors. Framed genes are commonly expressed in P and X tumors. Written in red are genes associated with disease progression; written in green are genes associated with favorable outcomes. Selection is adjusted p value < 0.05 and $Abs(logFC) > 1$.

Figure 10. Density of (A) tumor vessels and (B) tumor blood vessels with CD31/ α SMA colocalization. * and *, significantly increased vessel density in P and X tumors, respectively, as compared to CT; # and #, significantly decreased density of blood vessels with CD31/ α SMA colocalization in P and X tumors, respectively, as compared to CT; CT, control (non-irradiated cells).

Figure 11. cBioPortal data showing the correlation between VEGF-C over-expression and significantly lower (A) disease free and (B) overall survival rates in patients with HNSCC.

Figure 12. Quantification of hPDPN mRNA expression in long-term surviving CAL33 cells, selected *in vitro* after the third irradiation with either P or X (CR-MI setting).

Figure 13. Relative levels of VEGFC mRNA in different medulloblastoma (MDB) lines from different genetic groups: Sonic hedgehog cells (SHH), HDMB03 cells (Group 3) and Group 4 (cf. example 2 and reference 51).

Figure 14. VEGFC concentration in cells of Group 3 following exposition to various chemotherapies or following irradiation with 8 Gray photons.

The HDMB03 cells (Group 3 MDB) were incubated in the presence of various reference chemotherapies or irradiation with 8 Gray photons (CbPT: carboplatin, Eto,

etoposide, CT: vehicle, MIX, CbPT/Eto combination, Radio: irradiation, 8 grays). The chemotherapy concentrations correspond to the dose killing 50% of the cells (IC50). VEGFC was measured in cell culture medium by ELISA. ** $p < 0.01$; *** $p < 0.001$.

Figure 15. qPCR analysis of the different genes involved in the lymphatic program in HDMB03 cells (Group3 MDB); * $p > 0.05$; *** $p < 0.001$.

Figure 16. Immunohistochemistry (IHC) analysis of the presence of podoplanin labeled cells (stars) and structures (arrows) in three metastatic patients. The most aggressive MDB have circular structures that look like and that express a marker of lymphatic vessels.

EXPERIMENTAL PART:

EXAMPLE 1:

Material & Methods

Cell lines and culture

Two human HNSCC cell lines, CAL33 and CAL27, were provided through a Material Transfer Agreement with the Oncopharmacology Laboratory, Centre Antoine Lacassagne (CAL), where they had initially been isolated (50). The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 7% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA).

Cell irradiations

Five million cells were seeded onto 12 cm² tissue culture flasks, 48 h prior to the irradiations, which were carried out at CAL (four independent experiments) with either P (63 MeV Cyclotron MEDICYC, CAL, Nice, France) or X (6 MeV Dual energy Clinac 21EX Linear Accelerator, Varian Inc., Palo Alto, CA, USA). For clonogenicity assays, the cells were irradiated once (single irradiation, SI) with 1, 2, 4, 6 or 8 Grays (Gy; physical dose) and processed immediately after irradiation. To the purpose of all other experiments, the cells were irradiated either once or three times, one week apart (multiple irradiations, MI) with either 2 Gy (low dose) or 8 Gy (high dose), and processed 6h after irradiation. In the MI setting, cells were re-seeded after each irradiation and kept in culture until the next irradiation to reproduce the clinical situation where patients are usually given several irradiations. The chronic response (CR) was evaluated to determine if the changes associated with the acute response (AR) persist late (three weeks) after irradiation.

Two cell groups were thus generated from each independent irradiation experiment. They consisted of cells subjected to: (1) SI and analysis 48h thereafter (AR-SI); (2) MI and culture expansion (three weeks) after the third irradiation (CR-MI).

Clonogenicity assays

5 They were performed to quantify the radio-induced cell mortality, to generate the cell surviving curves, and to determine the relative biological effectiveness (RBE). Due to radiation dose-induced differences in plating efficiency, the cells were seeded at different densities: 3000 cells/dish for 0, 1, 2 and 4 Gy; 6000 cells/dish for 6 Gy, and 9000 cells/dish for 8 Gy. On day ten of culture, cells were stained for 20 minutes with Giemsa (Sigma
10 Aldrich, St. Louis, MO, USA). Stained plates were scanned and the number of cell colonies was determined with the Image J processing software (National Institutes of Health, Bethesda, MD, USA). The RBE was calculated as ratio of the biological effectiveness of P vs X irradiation, given the same dose/amount of absorbed energy (25).

Cell counting for viability and proliferation assessment

15 The cell counting for the CR-MI group was done every day, for four days post-seeding, in triplicate, with an automatic cell counter (Advanced Detection Accurate Measurement system, LabTech, TAMPA, FL, USA), according to the manufacturer instructions.

Quantification of gene expression

20 Molecular characterization of the irradiated cells was done by using the quantitative real-time – polymerase chain reaction. Total RNA was extracted with the RNeasy Mini Kit; first-strand cDNA synthesis was performed by using the QuantiTect® Reverse Transcription Kit (all from Qiagen, Hilden, Germany). cDNA samples were amplified by using the StepOnePlus™ RT-PCR System (Thermo Fisher Scientific) for 40 cycles with the Takyon™
25 Rox SYBR® Master Mix, dTTP Blue (Eurogentec, Liege, Belgium) and specific oligonucleotides (Sigma Aldrich), to assess mRNA expression for VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, VEGFR-3, NRP1, NRP2, IL-6, IL-8, CCL2, TRF2, PLK1, PD-L1, LYVE1, PDPN and PROX1. mRNA levels were normalized to a housekeeping mRNA coding for either the human or murine ribosomal protein, large, P0 (RPLP0). The
30 gene expression levels were given the individual scores of -1, 0 and 1 when they were significantly decreased, not significantly changed and significantly increased, respectively, as compared to control. For each irradiation setting, a global gene expression score was then calculated by cumulating the individual scores allocated to each gene expression level.

Protein quantification

VEGF-C protein was quantified by using an enzyme-linked immunosorbent assay (human DuoSet ELISA kit, R&D Systems, MN, USA). Protein concentration was normalized to the viable cell number.

5 Luciferase assays

CAL33 cells belonging to the CR-MI group were transfected by using 50 µl NaCl buffer, 1.25 µl of polyethylenimine transfection reagent (Sigma Aldrich) and 0.5 µg of total test plasmid DNA-renilla luciferase. The plasmids encoded either (i) a human vegf-c promoter fragment with either a non-mutated (wild type, WT) or a mutated (MUT) binding
10 site for the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (32), (ii) an artificial promoter containing three binding sites for human NF-κB or (iii) a human VEGF-C 3'UTR reporter (LightSwitch™, S803537, Active Motif, Carlsbad, CA, USA), all cloned downstream of the luciferase reporter gene. A CMV plasmid was used to control the variability of transfection efficiency in the reporter assays.

15 Tumor xenografts

The study was carried out in strict accordance with the recommendations of the United Kingdom Coordinating Committee on Cancer Prevention Research's Guidelines for the Welfare of Animals in Experimental Neoplasia. Our experiments were approved by the “Comité National Institutionnel d'Éthique Pour l'Animal de Laboratoire” (CIEPAL, reference:
20 NCE/2013-97). One million non-irradiated, P or X irradiated CAL33 cells (CR-MI group) were injected subcutaneously into the flank of 6-week-old NMRI-Foxn1nu/Foxn1nu female mice (Janvier Labs, Le Genest-Saint-Isle, France, n=10/group). The tumor volume ($v = L \times l^2 \times 0.52$) was determined following measurement with a caliper. When the tumors reached one cm³, the mice were sacrificed and the tumors collected.

25 Whole transcriptomic screening of tumor xenografts

For the sequencing and secondary analysis, 1 µg of total RNA was extracted from tumor xenografts, generated with either non-irradiated, P or X irradiated cells (n=3/group), by using the AllPrep® DNA/RNA/Protein Mini Kit (Qiagen). Lack of RNA degradation (ratio 28S/18S ≥ 1.6 and RIN > 7) was documented (Bioanalyzer 2100, Agilent Technologies, Santa
30 Clara, CA, USA). The libraries were generated by using Truseq Stranded mRNA kit (Illumina, San Diego, CA, USA). Libraries were then quantified with KAPA library quantification kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and pooled; 4nM of this pool were loaded on a Nextseq 500 high output flowcell and sequenced with a 2×75bp paired-end chemistry. STAR (2.4.0i) was used to map reads vs a STAR database containing:

Ensembl hg19 build (GRCh37.75), Ensembl mm10 build (GRCm38) and the ERCC spikes-in set, formatted with splice junctions information described from Ensembl release GRCh37.75 and GRCm38.83. STAR options were set to the recommended Encode RNA-seq options “--outFilterType BySJout --outFilterMultimapNmax 20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.04 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000”. Gene counts were obtained with featureCounts (subread-1.5.0-p3-Linux-x86_64) and “--primary -p -s 1 -C” options, by using the same GTF files used for STAR splice junctions training. Data were deposited in Gene Expression Omnibus (accession code GSE90761, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=opybisymbzotvkh&acc=GSE90761>).

For the heatmaps gene lists selection, genes involved in angiogenesis, inflammation, metastasis and cell proliferation were selected by using the Ingenuity Pathway Analysis (Qiagen) database. To define M1/M2 macrophages-related genes, the GEO dataset GSE69607 has been reanalyzed by using geo2R online resource. Genes up- and down-regulated (Abs (logFC)>2) in both M1 vs M0 and M2 vs M0 comparisons were selected as the “M1/M2 macrophages”-related gene list.

Histochemistry and immunofluorescence

Murine tumor sections were handled as previously described 8. To assess tumor architecture, the sections were subjected to Hematoxylin Eosin Saffron (HES) staining. For immunofluorescence, the frozen sections were incubated overnight, at 4°C, with the following primary antibodies: polyclonal rabbit anti-mouse/human LYVE1 (1:200; Abcam, Cambridge, United Kingdom), monoclonal mouse anti-mouse/human alpha smooth muscle actin (α SMA, 1:400, Sigma Aldrich) and monoclonal rat anti-mouse CD31 (1:50, clone MEC 13.3, BD Pharmingen, Heidelberg, Germany) primary antibodies, then incubated for 2h at room temperature, in the dark, with the secondary antibodies: anti-rabbit FP594, anti-mouse FP547 (1:1000, FluoroProbes, Interchim, Montluçon, France) and anti-rat AF488 (1:1000, AlexaFluor, Thermo Fisher Scientific); cell nuclei were stained with Hoechst (1:1000, Thermo Fisher Scientific). Cell and tissue preparations were examined under an inverted epifluorescence microscope (Axio Observer Z1) with an incorporated digital camera system for imaging (AxioCam Icc1); images acquisition and stitching, as well as the assessment of tumor vessels density, were performed with ZEN 2.3 software (all from Carl Zeiss MicroImaging GmbH, Weinheim, Germany).

Immunohistochemistry

Patient biopsy samples were collected with the approval of the local Ethics Committee, and their use in research was in accordance with the Declaration of Helsinki. The patient, disease, and treatment characteristics were described in the Table 3. Sections from formalin-fixed and paraffin-embedded biopsies from initial and relapsed tumors were incubated at room temperature with monoclonal, primary mouse anti-human PDPN and CD31 antibodies, as well as biotinylated secondary antibodies, by using an automated slide stainer (Ventana Medical Systems, Inc., Basel, Switzerland). Binding was detected with the diaminobenzidine substrate against a hematoxylin counterstain. Evaluation of marker expression was performed by an accredited clinical pathologist (I. P.).

Statistical analyses

Statistical analysis for all test, excepting whole transcriptomic screening data, was performed by two-tailed unpaired t test on at least three independent experiments; the results were considered statistically significant when p value < 0.05. For the whole transcriptomic screening, statistical analyses were conducted separately for human and mouse gene expression counts. Quality of libraries was assessed based on the Pearson correlation between observed vs expected ERCC counts ($R^2 > 0.90$ for all samples). Normalization and differential analysis were conducted within R/Bioconductor environment, by using DESeq2. P values were corrected for multiple testing, by using the Benjamini and Hochberg method. Heatmaps were generated with TMeV software. Heatmaps used the top 10 most up- and down-regulated genes, based on logFC and adjusted p value < 0.05 for human genes, and logFC only for mouse genes.

Tables

A mRNA levels (% of Control) - AR - SI		X		P	
Role	Gene	2 Gy	8 Gy	2 Gy	8 Gy
(Lymph)angiogenesis and metastasis	VEGF-A	132	*191	143	160
	VEGF-C	130	*227	159	*209
	VEGF-D	103	96	105	98
Inflammation	IL6	129	136	119	*#72
	IL8	147	*436	162	*401
	CCL2	104	123	#79	*136
Proliferation	TRF-2	88	*112	93	102
	PIK-1	85	*108	89	*99
Anti-tumor immunity	PD-L1	108	103	91	112
Gene score		4	6	0	3

B mRNA levels (% of Control) - CR - MI		X		P	
Role	Gene	2 Gy	8 Gy	2 Gy	8 Gy
(Lymph)angiogenesis and metastasis	VEGF-A	118	*208	#93	*#107
	VEGF-C	281	*626	226	#215
	VEGF-D	127	134	#95	#79
	VEGFR-1	96	*73	#72	73
	VEGFR-2	81	88	76	77
	VEGFR-3	116	91	100	80
	NRP1	105	90	#92	85
	NRP2	133	*169	#102	#129
Inflammation	IL6	120	126	#74	*#100
	IL8	87	*7916	#75	#90
	CCL2	112	*751	#83	#94
Proliferation	TRF-2	101	95	95	106
	PIK-1	91	97	89	*117
Anti-tumor immunity	PD-L1	119	140	#92	*116
Gene score		4	6	-8	-4

Table 1. Quantitative gene expression, as percentage of control (0 Gy), in either P or X irradiated CAL33 cells belonging to (A) AR-SI and (B) CR-MI groups. Highlighted values – significantly different (p<0.05) expression levels, as compared to control, for genes associated to favorable (dark grey) and non-favorable (black) outcomes; *, significantly different expression levels after low, as compared to high dose(s) of either P or X irradiation; #, significantly different expression levels after either low or high dose(s) of P, as compared to X irradiation.

Common up-regulated genes between X vs. 0 and P vs. 0		
Role	Gene abbreviation	Gene full name
Metastasis/ Angiogenesis	KRT16	keratin 16
	SERPINB3	serpin family B member 3
	CAPNS2	calpain small 2
	GRHL3	grainyhead like transcription factor 3
	CSTB	cystatin B
	PRSS27	protease, serine 27
	TLE4	transducin like enhancer of split 4
	TMPRSS11D	transmembrane protease, serine 11D
Inflammation	PGLYRP3	peptidoglycan recognition protein 3
	RASGRP1	RAS guanyl releasing protein 1
	ENDOU	endonuclease, poly(U) specific
	METRNL	meteorin like, glial cell differentiation regulator
	S100A8	S100 calcium binding protein A8
	S100A9	S100 calcium binding protein A9
	A2ML1	alpha-2-macroglobulin like 1
	HCN2	hyperpolarization activated cyclic nucleotide gated potassium channel 2
	CHST2	carbohydrate sulfotransferase 2
M1/M2	ABCG1	ATP binding cassette subfamily G member 1
Proliferation	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)
	BNIP1	BCL2 interacting protein like
	PPP2R2C	protein phosphatase 2 regulatory subunit Bgamma
	KLK8	kallikrein related peptidase 8
	GJB6	gap junction protein beta 6
	EEF1A2	eukaryotic translation elongation factor 1 alpha 2
	EPHA4	EPH receptor A4
	GAS7	growth arrest specific 7
	DSG1	desmoglein 1
	PDZK1IP1	PDZK1 interacting protein 1
	TMPRSS11A	transmembrane protease, serine 11A
	FLRT2	fibronectin leucine rich transmembrane protein 2

Other	C12orf36	putative uncharacterized protein C12orf36
	FRMPD1	FERM and PDZ domain containing 1
	TMEM45A	transmembrane protein 45A
	LIPK	lipase family member K
	CTC-490G23.2	CTC-490G23.2
	HOPX	HOP homeobox
	PLIN2	perilipin 2
	SDR9C7	short chain dehydrogenase/reductase family 9C, member 7
	STXBP5-AS1	STXBP5 antisense RNA 1
	ARRDC4	arrestin domain containing 4
	FRY	FRY microtubule binding protein
	FAM25A	family with sequence similarity 25 member A [
	SCEL	sciellin
	GJB2	gap junction protein beta 2
	UNC5B-AS1	UNC5B antisense RNA 1
	RP11-21B23.2	pre-mRNA processing factor
	SPRR1B	small proline rich protein 1B
	NAV3	neuron navigator 3
	SLC10A8	solute carrier family 10 member 8
	RP11-275I14.4	pre-mRNA processing factor
	RP11-358I2.4	pre-mRNA processing factor
	C9orf169	cysteine rich tail 1
	RP11-321G12.1	pre-mRNA processing factor
	LINC01094	long intergenic non-protein coding RNA 1094
	OR7E62P	olfactory receptor family 7 subfamily E member 62 pseudogene
	FAM3D	family with sequence similarity 3 member D
	SMIM5	small integral membrane protein 5
	FBXL16	F-box and leucine rich repeat protein 16
	RP11-783K16.5	pre-mRNA processing factor
	KCNK7	potassium two pore domain channel subfamily K member 7
	FAM25HP	family with sequence similarity 25, member H pseudogene
	WI2-85898F10.1	uncharacterized LOC107985535
	IVL	involucrin
	TCN1	transcobalamin 1
	KLHL4	kelch like family member 4
	LRRC7	leucine rich repeat containing 7
	RP11-557H15.3	pre-mRNA processing factor
	TSHZ2	teashirt zinc finger homeobox 2
	OLFM2	olfactomedin 2
	ADH7	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide
Common down-regulated genes between X vs. 0 and P vs. 0		
Role	Gene abbreviation	Gene full name
Proliferation	LIF	leukemia inhibitory factor
Other	RNA5-8SP2	RNA, 5.8S ribosomal pseudogene 2
	P2RX5	purinergic receptor P2X 5

Table 2. Common up-regulated and down-regulated human genes in tumors generated with either X or P irradiated cells. In bullet are genes up-regulated in either P or X tumors, but down-regulated in tumors generated with non-irradiated cells. Selection is adjusted p value < 0.05 and lofFC > 1.

#	Patient	Diagnosis	Disease stage	Treatment	Disease progression	Time to 1 st relapse
1	72-year old woman	Oral SCC (2011)	T4N2M0	Radical surgery; adjuvant X radiotherapy (50 Gy)	Loco-regional relapses (2012, treated with chemotherapy and stereotactic radiotherapy); relapse and death (2013)	14.2 months
2	63-year old man	Hypo-pharyngeal SCC (2008)	T3N2bM0	2 induction chemotherapy cycles (cipistin-5FU-docetaxel); total pharyngolaryngectomy; adjuvant platinum-based chemotherapy and X radiotherapy (64 Gy)	Loco-regional relapses (2010 and 2012, treated by surgery); metastases (2013); death (2014)	21 months
3	74-year old man	Hypo-pharyngeal SCC (2011)	T2N2bM0	Pharyngolaryngectomy; adjuvant X radiotherapy (66 Gy)	Loco-regional relapse and metastases (2012); death (2015)	19.2 months

Table 3. Diagnosis, disease and treatment characteristics of patients with HNSCC.

mRNA levels (% of Control) - CR - MI		X	P
Role	Gene	8 Gy	8 Gy
(Lymph)angiogenesis and metastasis	VEGF-A	154	#108
	VEGF-C	205	#152
	VEGF-D	279	189
	VEGFR-1	76	60
	VEGFR-2	76	88
	VEGFR-3	54	44
	NRP1	283	#155
	NRP2	161	164
Inflammation	IL6	257	#67
	IL8	305	316
	CCL2	24	#68
Proliferation	TRF-2	92	96
	Plk-1	95	107
Anti-tumor immunity	PD-L1	148	190
Gene score		5	1

Table 4. Quantitative gene expression, as percentage of control (0 Gy), in either P or X irradiated CAL27 cells belonging to CR-MI group. Highlighted values – significantly different ($p < 0.05$) expression levels, as compared to control, for genes associated to anti-tumor (dark grey) and pro-tumor (black) effects; *, significantly different expression levels

after 2 Gy, as compared to 8 Gy of either P or X irradiation; #, significantly different expression levels after either 2 Gy or 8 Gy of P, as compared to X irradiation.

Results

Cell survival/proliferation is in favor of P following single irradiation, and X following multiple irradiations

Our hypothesis was that irradiation would lead to different cell viability and proliferation profiles depending on the radiation type and dose, number of irradiations, and time of assessment. We qualified as the “acute response (AR)” the modifications of biological parameters (proliferation, survival, gene expression) a few hours following a single irradiation (SI). The modifications of the same biological parameters on cells that have survived to multiple irradiations (MI) and that have been expanded as new populations were qualified as the “chronic response (CR)”.

In order to calibrate our experiments, we first determined a relative biological effectiveness (RBE) of photons and protons on our model cell lines following SI. According to the literature, P therapy treatments are based on a RBE of 1.1, relative to high-energy X therapy (25). The surviving curve of CAL33 cells following administration of escalating doses of either P or X irradiation confirmed a RBE of 1.1 for P, as compared to X irradiation (Figure 6). This experiment confirms the literature data showing that P kills tumor cells more efficiently than X irradiation (25).

However, patients are irradiated several times to reach a maximal therapeutic efficacy. Therefore, our next purpose was to compare the relative aggressiveness of cells that were resistant to MI by X or P. Hence, we performed our experiments on two independent cell lines (CAL33 and CAL27). The proliferative ability along a time course of CAL33 (Figure 1) or CAL27 (Figure 7) that have survived to MI was determined. As compared to non-irradiated cells, the proliferation of X or P irradiated cells was reduced in both models and the difference was striking 96h following cell seeding ($p<0.001$ for CAL33; $p=0.049$ for CAL27). However, the difference in proliferation became statistically significant earlier for X irradiated cells in the CAL33 model ($p=0.02$ for X8 at 48h; $p=0.014$ and 0.009 for X2 and X8, respectively, at 72h; $p<0.001$ for all conditions at 96h). Whereas the difference in proliferation did not reach statistical significance between X2 and X8 irradiated cells, X8 cells proliferated to a lesser extent, as compared to P2 and P8, at 48h post seeding ($p=0.006$ and $p=0.035$, respectively), to P2 at 72h post seeding ($p=0.018$), and P2 and P8 irradiated cells at 96h post seeding ($p=0.012$ and $p=0.008$, respectively).

Therefore, the overall therapeutic advantage, attested by reduced cell viability and proliferation capacity following SI switched in favor of X post MI for CAL33 cells. For CAL27, no difference in the proliferative ability of MI X and P cells was observed, suggesting that X and P exert different outcomes, depending on the HNSCC type.

P irradiation leads to overall lower induction of mRNA coding pro-inflammatory, pro-(lymph)angiogenic and pro-proliferative genes

The gene expression levels for CAL33 cells following SI or MI, represented as percentage of control, and the gene expression scores are listed in the Table 1 A and B. The mRNA levels of the different tested genes overall increased in a dose-dependent manner and with the irradiation number after both P and X irradiation. Genes involved in (lymph)angiogenesis, inflammation and immune tolerance were overall less expressed after high dose(s) of P, as compared to X, irradiation in all investigated groups; the genes involved in (lymph)angiogenesis, inflammation and immune tolerance were down-regulated after P irradiation, showing significantly lower mRNA levels, as compared to X irradiation, within the following settings: (i) AR-SI after low dose: CCL2 ($p=0.035$) and high dose: IL-6 ($p=0.0001$); (ii) CR-MI after low dose: VEGF-A ($p<0.0001$), IL-6 ($p<0.0001$), IL-8 ($p=0.046$), CCL2 ($p=0.041$), PD-L1 ($p=0.002$) and high dose: VEGF-D ($p<0.0001$) and IL-8 ($p<0.0001$). By contrast, among these genes, X irradiation led to down-regulation of IL-8 only, within the low dose CR-MI settings. Notably, VEGF-C mRNA levels were systematically increased after both P and X irradiation, but they were significantly lower after P, as compared to X irradiation, after high dose within the CR-MI setting ($p<0.001$). Among all investigated genes, IL-8 was the gene whose mRNA was induced at the highest level after X (79-fold, as compared to control), but not after P irradiation, within the high dose CR-MI setting ($p<0.0001$). Moreover, both P and X irradiation augmented PD-L1 mRNA expression in a dose-dependent manner within the AR-SI and CR-MI settings, and in an irradiation number-dependent manner within the AR-SI setting. The generated gene expression scores showed that P irradiation is associated with a more favorable profile [reduced proliferation, (lymph)angiogenesis, inflammation]. A similar gene score, in favor of P irradiation, was also obtained for CAL27 cells, within the CR-MI setting, despite of an increase in VEGF-C, VEGF-D, NRP1, NRP2, IL-8 and PD-L1 mRNA expression (Table 4).

Induction of VEGF-C protein is reduced in P irradiated cells

Because lymph node metastasis is frequent at diagnosis in HNSCC and in patients who relapse locally after radiotherapy, we focused our research on VEGF-C, the major growth factor for lymphatic endothelial cells. Although the mRNA levels of VEGF-C were

increased after both low and high dose(s) of P or X irradiation, they were lower after high dose(s) of P irradiation. To confirm the results obtained at mRNA level, we next assessed VEGF-C protein levels in CAL33 and CAL27 cells.

5 In CAL33 cells, VEGF-C protein levels increased in a dose-dependent manner following both P and X irradiation. Furthermore, they were significantly lower after P irradiation. Within the AR-SI setting (Figure 2A), VEGF-C protein levels were significantly increased after a low and high dose of irradiation with either P ($p=0.038$ and $p=0.046$, respectively) or X ($p=0.0002$ for both dose types). A significantly lower expression was observed after a high dose of P, as compared to X irradiation (by 59%, $p=0.018$). However, 10 significantly increased levels were observed after a high vs low dose of X irradiation (3-fold increase, $p=0.002$).

The VEGF-C protein induction was also maintained at significantly increased levels in CAL33 cells of the CR-MI group (Figure 2B), after both low and high doses of P and X irradiation ($p<0.001$), with significantly decreased levels after high doses of P vs X irradiation 15 (by 50%, $p<0.001$). In addition, there were significantly increased levels after high, as compared to low doses of X irradiation ($p=0.002$). These observations were confirmed in CAL27 cells within the CR-MI setting (Figure 7B), where VEGF-C protein levels were significantly increased after both P and X irradiations ($p<0.001$), with lower levels after high doses of P vs X irradiation ($p=0.001$).

20 **X and P irradiations stimulate the VEGF-C promoter activity**

Irradiation by either X or P stimulated the activity of the vegf-c promoter especially in CAL33 cells surviving to multiple X irradiations (6- and 18-fold increase, respectively, $p<0.001$, Figure 2C). This result is consistent with the induction of the VEGF-C mRNA within the CR-MI setting (Table 1) and suggests a chronic induction of vegf-c gene 25 transcription, an increase in vegf-c mRNA half-life or a combination of both mechanisms. Mutation of the NF- κ B binding site (MUT) had no effect on the basal vegf-c promoter activity in non-irradiated cells. However, in cells surviving to MI by P and X, the activity of the MUT, as compared to WT, promoter was significantly decreased (by 33%, $p=0.004$ and by 30%, $p=0.027$, respectively, Figure 2C) suggesting that the increase in the transcriptional 30 activation of the vegf-c promoter depends in part on a constitutive activation of NF- κ B. In the CAL27 cell line, the irradiation by either P or X did not stimulate the activity of the WT vegf-c promoter but the activity of the MUT promoter was completely inhibited in both non-irradiated and irradiated cells ($p<0.001$, Figure 7C). To further assess the role of NF- κ B on vegf-c promoter, the activity of an artificial promoter containing three binding sites for human

NF- κ B was determined in control and irradiated cells. In CAL33, the NF- κ B-dependent promoter activity was lower in P irradiated cells, which is consistent with the activity of the vegf-c promoter having a WT NF- κ B binding site (Figure 2D). For CAL27, the NF- κ B-dependent promoter activity is almost equivalent in control and either X or P irradiated cells (Figure 7D). This result indicates that the vegf-c promoter activity exclusively relies on a NF- κ B-dependent transcriptional mechanism in CAL27 cells, whereas the dependency to NF- κ B is partial in CAL33 cells. Moreover, a reporter gene used to assess VEGF-C mRNA half-life was not affected by either P or X irradiation in CAL33 cells (Figure 2E), suggesting that the increase in vegf-c mRNA levels does not depend on modifications in mRNA half-life.

Cells surviving multiple irradiations by P and X generate tumors with distinct characteristics

The cells resistant to MI by either P or X served to generate experimental tumors in mice to test their relative aggressiveness. The average tumor volume was significantly increased ($p < 0.05$) for P and X tumors, but no differences were observed between the irradiation types (Figure 3A and 3.B). These results were inconsistent with the *in vitro* proliferative abilities of the cells surviving after MI with either P or X. To determine whether P and X irradiated cells “educated” the microenvironment to favor tumor growth, we performed a whole transcriptomic screening of the tumors. Indeed, distinct profiles for both the mouse (Fig. 3.C) and human (Fig. 3.D) ten most up- and down-regulated genes were detected. Among the ten most up- and down-regulated mouse genes, some (Fig. 3.C) such as collagen type XVII alpha 1 and carbonic anhydrase 2 (Car2)26 had a shared pattern of expression in P and X tumors (Fig. 3.C). In addition, we identified distinct profiles for the ten most up- and down-regulated mouse (Figure 8) and human (Figure 9) genes involved in angiogenesis, inflammation, metastasis, M1/M2 macrophage transition. Some of these genes had a shared pattern of expression in P and X tumors.

Furthermore, we identified 70 (26%) common up-regulated and 3 (5.8%) common down-regulated genes (Fig. 3.E) between X and P tumors, with roles in angiogenesis/metastasis, inflammation, M1/M2 macrophage transition and proliferation (Table 2).

Tumors induced by irradiated cells presented less necrosis and increased intra-tumor vessels density ($p = 0.031$ for P and $p = 0.002$ for X group, Fig. 4.A and Figure 10A). In addition, irradiation by either P or X led to generation of tumors with destabilized vessel architecture (Fig 4.B), attested by a decrease in vessels with co-staining for CD31 and α SMA ($p = 0.005$ for P and $p = 0.006$ for X group, Figure 10B). Lymphatic vessels were detected in the

tumor-skin border of the control and P groups (Fig. 4.C). However, they were also present in the core of the X tumors, finding consistent with the over-expression of VEGF-C observed *in vitro*. Since VEGF-C was particularly discriminative between the two experimental irradiation conditions, we tested whether it had induced the development of lymphatic vessels.

LYVE1, PDPN and PROX1 markers of lymphatic vessels were then tested (Fig. 4.D). LYVE1 and PDPN mRNA were down-regulated ($p < 0.001$ for both markers) in P and up-regulated ($p = 0.015$ and 0.044 , respectively) in X tumors. Lower mRNA levels of LYVE1 and PDPN were detected in P, as compared to X tumors ($p = 0.003$ and $p < 0.001$, respectively). PROX1 mRNA level was down-regulated ($p = 0.02$) and unchanged in P and X tumors, respectively.

Conventional radiotherapy by X increases tumor lymphangiogenesis in patients with HNSCC

To further correlate the relationship between irradiation-dependent VEGF-C expression and lymphatic vessels development, we tested the presence of lymphatic markers in biopsies from primary and locally relapsed human HNSCC, after conventional radiotherapy. Recent reports described that the expression of PDNP, one of the major makers of lymphatic vessels, was not restricted to lymphatic vessels but it was also expressed in HNSCC cells. Expression of PDPN was indeed detected in tumors from patients with oral and pharyngeal SCC (Fig. 5.A – 1a, 2a). However, we observed a high increase of PDNP labeling, in both tumor and lymphatic cells, in sections from relapsed tumors after treatment with conventional X radiotherapy (Fig. 5.A – 1b, 2b). In the same tumors, the vascular network, attested by CD31 labelling, was not modified in the relapsed (Fig. 5.B – 1b, 2b), as compared to the initial tumors (Fig. 5.B – 1a, 2a). In addition, a tendency for increased mRNA expression of PDPN ($p = 0.088$), along with significantly increased mRNA expression of VEGF-C ($p = 0.005$), LYVE1 ($p = 0.025$) and PROX1 ($p = 0.003$) were detected in relapsed patient tumors after conventional X radiotherapy (Figure 5C).

Discussion

Our *in vitro* results indicate that P irradiation led to lower expression of factors involved in (lymph)angiogenesis, inflammation and immune tolerance. This suggests the acquisition of less aggressive phenotypes after P therapy. The selection of surviving cells was still possible after MI, indicating a mechanism of acquired resistance secondary to irradiation (28). However, the molecular profiling of the surviving cells suggests a more aggressive *in vivo* phenotype after MI with X. Therefore, due to its physical and biological properties, P irradiation may be more efficient in tumor size control through dose escalation.

The long-term surviving cells after three irradiations with P showed a down-regulation of the investigated pro-angiogenic/pro-inflammatory genes, except for vegf-c, while most of these genes were up-regulated after X irradiation. The implication of VEGF-C in the metastatic dissemination process after irradiation has not been elucidated. To our knowledge, this is the first report showing P or X radiation-induced VEGF-C over-expression at both gene and protein levels in HNSCC cells. The VEGF-C mRNA levels increased in a dose-dependent manner and with the irradiation number, except in the cells surviving after three irradiations with P. These observations suggest that P radiotherapy would lead to less pronounced lymphangiogenesis/metastasis, as compared to X radiotherapy.

Therefore, we postulated that over-expression of VEGF-C may represent an extrinsic mechanism responsible for the post-irradiation tumor dissemination/metastasis in HNSCC. VEGF-C expression was associated with lymph node metastasis, recurrence and a poorer five-year survival rate in patients with HNSCC, being an independent prognostic factor (11, 29). Moreover, the online available database cBioPortal (<http://www.cbioportal.org>) shows that over-expression of VEGF-C correlated to significantly lower disease free ($p=0.0022$, Figure 11A) and overall ($p=0.015$, Figure 11B) survival rates in patients with HNSCC ($n=517$). It has been reported that gamma rays irradiation induced VEGF-C expression and endothelial cell proliferation in lung cancer (30). These observations, corroborated with ours, suggest that VEGF-C may be an important therapeutic target for HNSCC patients who relapse after radiotherapy with either P or X.

Because VEGF-C might be a major factor responsible for post-irradiation disease progression in HNSCC patients, via promotion of lymphangiogenesis, we further started investigating the mechanisms involved in its induction, which may serve to its therapeutic targeting. Regulation of VEGF-C expression has been poorly addressed (27, 31, 32). Irradiation-mediated induction of VEGF-C mRNA suggested stimulation of transcription, stabilization of mRNA or a combination of these mechanisms (31). Our data indicate that both P and X irradiation stimulated the activity of a short form of vegf-c promoter in CAL33 cells. The vegf-c promoter contained a binding site for NF- κ B. The dependency of this site is variable considering the two cell lines we tested, but nevertheless NF- κ B plays a key role in VEGF-C regulation, as suggested in another cancer type (32). As these cell lines came from two different patients, our results highlight the inter-patient variability in VEGF-C expression and regulation, stressing out the importance of implementing personalized diagnosis and treatment strategies.

In the cells surviving after three irradiations, the VEGF-A and VEGF-D genes were down-regulated by P and up-regulated by X irradiation. VEGF-A expression significantly correlated with lymph node metastasis in patients with HNSCC 11. High VEGF-A expression was also associated with higher clinical stages and worse overall survival, being a significant predictor of poor prognosis in patients with HNSCC (33). Furthermore, VEGF-D expression correlated with lymphatic vessel density and lymph node metastasis in these patients (10). In addition, VEGFR-2, VEGFR-3 and NRP1, highly expressed by HNSCC cells (34), were down-regulated in the surviving cells selected after three irradiations with P, but not with X. High NRP1 and NRP2 levels correlated with poor prognosis in HNSCC patients, NRP2 being an independent prognostic markers for overall survival (35).

Therefore, our study sets the basis for clinical assays investigating more efficient treatments, combining P radiotherapy with anti-angiogenic targeted therapies. Such combinations would eventually lead to decreased selection of post-irradiation surviving cells and lower relapse rates in patients with HNSCC, for which the current treatments include X irradiation (3). A case report describing the successful treatment of a patient with chondrosarcoma by combining P radiotherapy with sunitinib, an inhibitor of VEGFRs and platelet-derived growth factor receptor, underlines the effectiveness of such approach (36).

We also showed that P and X radiations differently modulated the pro-inflammatory gene expression in HNSCC cells. Among the assessed genes, the highest determined mRNA level was for IL-8. Stress and drug-induced IL-8 signaling conferred chemotherapeutic resistance to cancer cells (37). Serum and tumor IL-8 significantly affected the disease free survival in patients with early stage HNSCC (38). Therefore, inhibiting the effects of IL-8 signaling in combination to chemoradiotherapy may be of significant therapeutic value.

P but not X irradiation down-regulate IL-6 expression at the mRNA level. IL-6 expression predicted a poor response to radio-chemotherapy and a non-favorable prognosis in HNSCC patients (39). It was also linked to radiation resistance and development of chronic toxicities after irradiation (40). Depending on tumor location, the most common side effects after conventional radiotherapy of HNSCC include mucositis, xerostomia, dysphagia requiring short-term or permanent gastrostomy, soft tissue/bone necrosis, neck fibrosis, and thyroid dysfunction (41). Although the primary goal in radiotherapy is tumor control, a parallel essential goal is to spare normal tissues from radiation toxicity. Therefore, our data bring further pre-clinical evidence that the use of P irradiation in the treatment of HNSCC may lead to less inflammatory side effects.

We also showed that, in the cells surviving long-term after three irradiations, another major pro-inflammatory cytokine, CCL2, was down-regulated after P, while being highly up-regulated after X irradiation. As serum CCL2 levels were associated with HNSCC progression (42), our data suggest that P therapy might be more beneficial for these patients.

Our results also showed that PLK1 and TRF2 genes were differently regulated after P or X irradiation and correlated to the proliferation patterns. By inhibiting apoptosis, PLK1 over-expression was associated with poor survival in patients with HNSCC, being an independent prognostic factor (43). Its targeting with a multi-kinase inhibitor led to encouraging anti-tumor activity in patients with SCC (44). These data suggest that PLK1 might be a potential therapeutic target for HNSCC patients undergoing radiotherapy. TRF2 may also become an established predictive marker for treatment efficacy and a marker of survival in HNSCC. We previously showed that the treatment response was increased in TRF2 knocked-down cells and that TRF2 over-expression had a negative impact on patients' survival (23).

Irradiation leads to adaptive changes in the tumor microenvironment that may limit the generation of an anti-tumor immune response (24). Indeed, we showed a significant increase of PD-L1 expression after P, and confirmed the X radiation-induced PD-L1 expression in other cancers (24, 45). In patients with HNSCC, high PD-L1 expression in primary tumors correlated with metastasis and poor prognosis, being an independent prognostic factor (46). PD-L1 was also a significant predictor for poor treatment response and shorter survival in X radiotherapy-treated patients with HNSCC (45). A phase II, multi-center, single-arm, global study of monotherapy with durvalumab, a Fc optimized monoclonal antibody directed against PD-L1, is ongoing in our institution in patients with recurrent/metastatic HNSCC and PD-L1 positive status. Therefore, our data, associated to the progress in the field, set the basis for the investigation of novel therapeutic strategies for HNSCC, based on the PD-L1 – PD-1 interaction, in combination with radiotherapy.

We also demonstrated that the aggressiveness of the irradiated cells was augmented *in vivo* through increased tumor volume, density of tumor vessels and blood vessels with destabilized architecture. These observations suggest that the irradiation-adapted cells have acquired different transcriptome and secretome profiles. Indeed, among the common human genes up-regulated in either X or P tumors, but down-regulated in tumors generated with non-irradiated cells, we identified PDZK1 interacting protein 1 (PDZK1IP1, known also as MAP17) (47) and fibronectin leucine rich transmembrane protein 2 (FLRT2) (48), known for promoting cell proliferation. In addition, mouse Car2 expression was down-regulated in P and

X tumors, while up-regulated in tumors generated with non-irradiated cells. Interestingly, low CAR2 protein expression has been associated with increased tumor size (26). In addition, the X tumors showed up-regulation of human genes involved in metastasis, angiogenesis and epithelial mesenchymal transition, such as MMP2, MMP9, MMP13, MMP16, MMP28 and vimentin15, while P tumors showed up-regulation of human C-C Motif Chemokine Ligand 5 chemokine gene involved in CD8⁺ T lymphocytes recruitment associated with better clinical outcomes (49).

To get further insights whether tumor cell adaptation following radiotherapy may contribute to clinical disease progression, in part through lymphangiogenesis, we investigated lymphatic markers expression in patients with relapsed HNSCC after X radiotherapy. Biopsies at relapse are very rarely sampled in radiotherapy-treated patients. However, in this small cohort, all patients presented increased protein and/or mRNA levels of PDPN, VEGF-C, LYVE1 and PROX1, bringing evidence that conventional radiotherapy may promote lymphangiogenesis. It has also been reported by others that high PDPN expression is associated with aggressive tumor behavior, poor prognosis and metastatic regulation through interaction with VEGF-C, suggesting that PDPN may be used as a potential prognostic biomarker for HNSCC (27). However, our *in vitro* studies did not reveal increased PDPN expression in HNSCC cells that resisted to MI (Figure 12).

In conclusion, our study highlighted the differential gene/protein expression profile after P vs X irradiation in HNSCC and potential candidate markers for prognosis, efficacy of anti-tumor treatments and new anti-tumor targets, such as VEGF-C. Beside the physical advantage of P irradiation in dose deposition, our observations provide preclinical evidence that beam therapy with P might be superior to conventional X therapy in HNSCC patients, due to its biological advantages. P irradiation could therefore permit dose escalation without increasing the side effects, while increasing the tumor control. Further work is also needed to refine the strategies for blocking VEGF-C activity and its effects on the vascular/lymphatic endothelial or tumor cells with anti-angiogenic therapies. The implementation of P therapy in combination with anti-angiogenic or anti-immune checkpoint drugs for HNSCC will therefore require prospective randomized clinical trials to measure the toxicity and disease control.

EXAMPLE 2:

Medulloblastomas (MDBs) are fast-growing tumors that belong to the group of primitive neuro-ectodermal tumors of the central nervous system. 70% of MDBs are diagnosed before the age of 10 and MDB are rare in adults. MDB is a pathology composed of

four molecular groups: wingless (WNT), sonic hedgehog (SHH), group 3 and group 4 (51). These groups are defined by clinical and molecular parameters of the tumor cells. The WNT and SHH groups exhibit aberrant activation of the WNT and SHH signaling pathways. Group 3 tumors overexpress the *OTX2* and *c-MYC* genes and those of group 4 *N-MYC* (52). The genetic landscape of MDB more precisely describes and illustrates tumor heterogeneity among the previously identified subgroups (53-55). These classifications represent important indicators for treatment decision at the first line but do not predict patients at risk of relapse after conventional therapies. MDB treatments combine surgery, irradiation and/or multiple chemotherapies (carboplatin, etoposide). These heavy treatments induce physical, psychological or behavioral sequelae. MDBs are highly vascularized by overexpression of VEGF and many other markers of angiogenesis (VEGFB, VEGFC, FGF, angiopoietin) (56). Anti-angiogenic treatments induce a poor response rate in MDB and are not devoid of side effects in developing children. Innovative treatments use inhibitors of the SHH pathway (vismodegib) (57) and a precise radiotherapy by proton which saves healthy tissues (58). Physicians claimed that 70% of the children are cured but in case of relapse the issue is fatal in 100% of cases. Predicting such fatal relapses as precisely as possible represents a very important therapeutic issue.

Material & Methods

Cell culture

DAOY and HDMB03 cell lines were purchased from the ATCC. Stocks were made at the original date of obtaining the cells, and were usually passaged for no more than 4 months. These cell lines have been authenticated by DNA profiling using 8 different and highly polymorphic short tandem repeat loci (DSMZ).

ELISA test

Cell supernatant was recovered for VEGFC measurement using the Human DuoSet ELISA kit (R&D Systems).

Quantitative real-time PCR experiments

One microgram of total RNA was used for the reverse transcription, using the QuantiTect Reverse Transcription Kit (Qiagen), with blend of oligo (dT) and random primers to prime first strand synthesis. SYBR Master Mix Plus (Eurogentec) was used for quantitative real-time PCR (qPCR). The mRNA level was normalized to 36B4 mRNA.

Oligo sequences:

	Forward	Reverse
36B4	CAGATTGGCTACCCAACTGTT	GGCCAGGACTCGTTTGTACC
LYVE1	TGTCAAAGGTATGTGAAGG	TCTAAACAGCTTCCAGG
NRP2	GCTGGCTATATCACCTCTCCC	TCTCGATTTCAAAGTGAGGGTTG
PROX1	AGTTCAACAGATGCATTACC	TCTCTGGTTATAGACAGCTC
PDN	AAGATGGTTTGTCAACAGTG	GTACCTTCCCACATTTTTC
VEGFC	TTACGGTCTGTGTCCAGTGTA	TTCTCTGTTATGTTGCCAGCC
VEGFR3	TGCACGAGGTACATGCCAAC	GCTGCTCAAAGTCTCTCACGAA

Gene expression microarray analysis

Normalized RNA sequencing data produced by The German Cancer Center Consortium and the “Genomics Analysis and visualization platform” is available at the following address:

https://hgserver1.amc.nl/cgi-bin/r2/main.cgi?&dscope=MB500&option=about_dscope.

Almost 200 samples were analyzed.

Immuno-histo chemistry

Samples were collected with the approval of the Local Ethics committee of Nice. Sections from blocks of formalin-fixed and paraffin-embedded tissue were examined for immunostaining for podoplanin. After deparaffinization, hydration, and heat-induced antigen retrieval, the tissue sections were incubated for 20 minutes at room temperature with monoclonal anti-podoplanin antibodies diluted at 1:100. Biotinylated secondary antibody (DAKO) was applied and binding was detected with the substrate diaminobenzidine against a hematoxylin counterstain.

Results**Patients of Group 3 MDB express highest levels of VEGFC.**

Analysis of online available data shows that the most aggressive Group 3 subgroup expressed the highest level of VEGFC (Figure 13).

Chemo/radiotherapeutic treatments result in the expression of VEGFC and several lymphatic markers in a Group 3 MDB model cell line.

The standard chemotherapeutic treatments for MDB are combinations of carboplatin/etoposide. We determined the IC50 for each of these compounds on different

MDB lines. Our results show VEGFC induction by chemotherapies/irradiation in MDB cells (Figure 14).

We also observed that expression of different markers of lymphatic cells including VEGFR3, NEUROFILIN 2 and more importantly PROX1, a master transcription factor for the development of the lymphatic system (59, 60) was stimulated by photon based radiotherapy (Figure 15). We hypothesize that in some tumors or in response to treatments tumor cells may dedifferentiate to lymphatic cells allowing the development of hybrid LV equivalent to those described for vasculomimicry (61). We called this phenomenon “Lymphomimicry”.

Presence of cells labelled with a lymphatic marker (podoplanin) in MDB samples

We believe that the presence of lymphatic vessels (LV) is a marker of aggressiveness of tumors and may guide the treatment. Aggressive treatment should only be given in the most severe cases. Limiting a radio/chemo heavy treatment would reduce the toxic side effects. The presence of LV in the brain is a real debate. Pathologists advocate the absence of such vessels. However, their presence has been recently identified in the meninges (62, 63). The presence of LV in brain tumors has never been reported. We have observed and herein disclose for the first time the presence of cells and/or structures revealed by podoplanin labeling (commonly used by pathologist to identify lymphatic embols) in aggressive MDB cases (Figure 16). We believe these structures to be lymphatic vessels. We further believe that patients exhibiting such structures should benefit from an aggressive treatment of cancer, typically radiotherapy, preferably P radiotherapy, and/or chemotherapy using a VEGF-C inhibitor as the chemotherapeutic agent.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS

1. A method for predicting the outcome of a cancer in patient afflicted with solid cancer after radiotherapy treatment, comprising the steps of: i) determining the expression
5 level of VEGF-C in a biological sample obtained from said patient, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the patient has a good prognosis when the level determined at step i) is lower than the predetermined reference value or concluding that the patient has a poor prognosis when the level determined at step i) is higher than the predetermined
10 reference value.
2. The method according to claim 1, wherein the cancer is a head and neck squamous cell carcinoma (HNSCC).
3. The method according to claim 1, wherein the cancer is a medulloblastoma (MDB).
4. The method according to any one of claims 1 to 3, wherein the radiotherapy treatment
15 is photon (X) radiotherapy.
5. The method according to any one of claims 1 to 3, wherein the radiotherapy treatment is proton (P) radiotherapy.
6. A VEGF-C inhibitor for use in the treatment of solid cancer in a patient in need thereof, wherein the patient was being classified as having a poor prognosis by the
20 method according to any one of claims 1 to 5.
7. The VEGF-C inhibitor for use according to claim 6 in combination with radiotherapy treatment.
8. The VEGF-C inhibitor for use according to any one of claims 6 or 7, wherein said VEGF-C inhibitor is a small organic molecule, a polypeptide, an aptamer, an antibody,
25 an oligonucleotide, a ribozyme or a CRISPR.
9. The VEGF-C inhibitor for use according to any one of claims 6 or 7, wherein said VEGF-C inhibitor is an anti-VEGF-C antibody such as VGX-100, VC4.5, and VC1.12.

10. The VEGF-C inhibitor for use according to any one of claims 6 or 7 in combination with an anti-cancer compound or an anti-angiogenic compound.
11. The VEGF-C inhibitor for use according to claim 10, wherein said anti-angiogenic compound is a tyrosine kinase receptor (TKR) inhibitor such as sunitinib, vandetanib, pazopanib, sorafenib and cediranib.
12. A method for treating solid cancer in a patient in need thereof wherein the patient was being classified as having a poor prognosis by the method according to any one of claims 1 to 5, comprising the step of administering to said patient a VEGF-C inhibitor in combination with radiotherapy treatment.

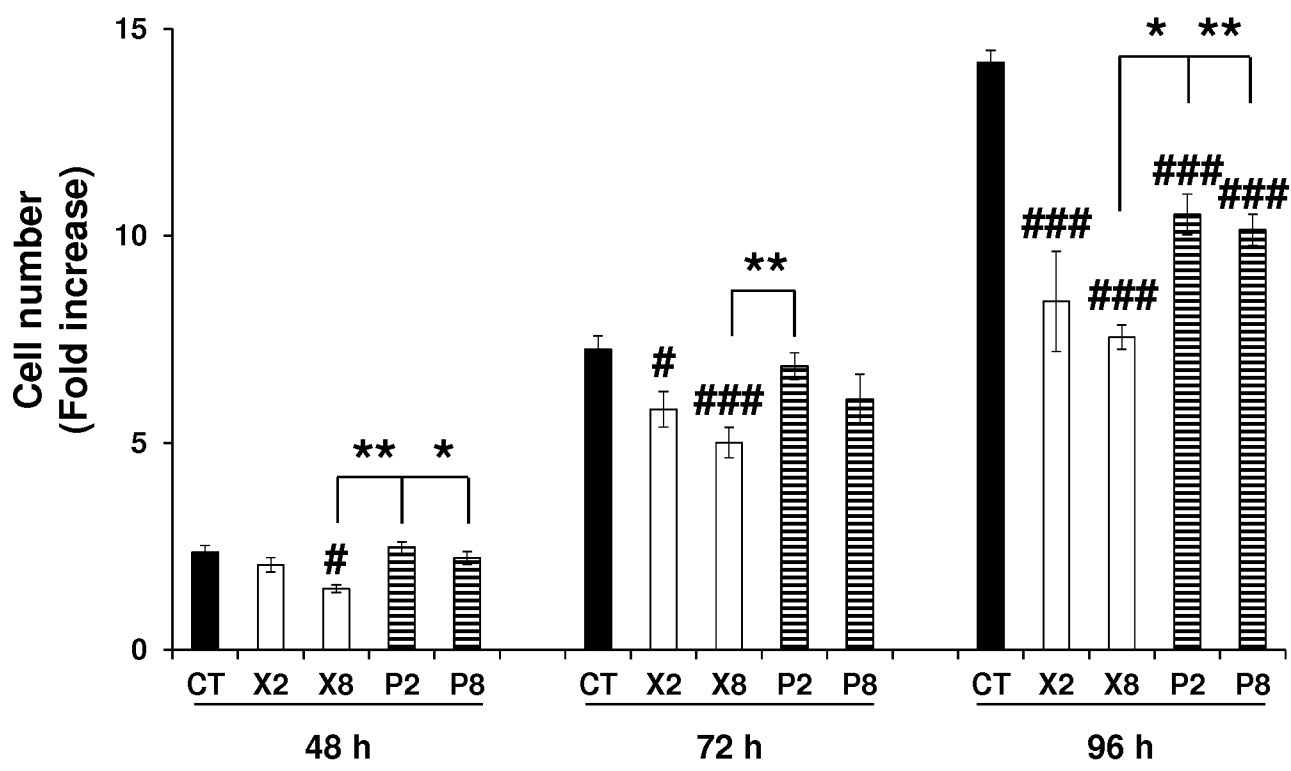


FIGURE 1

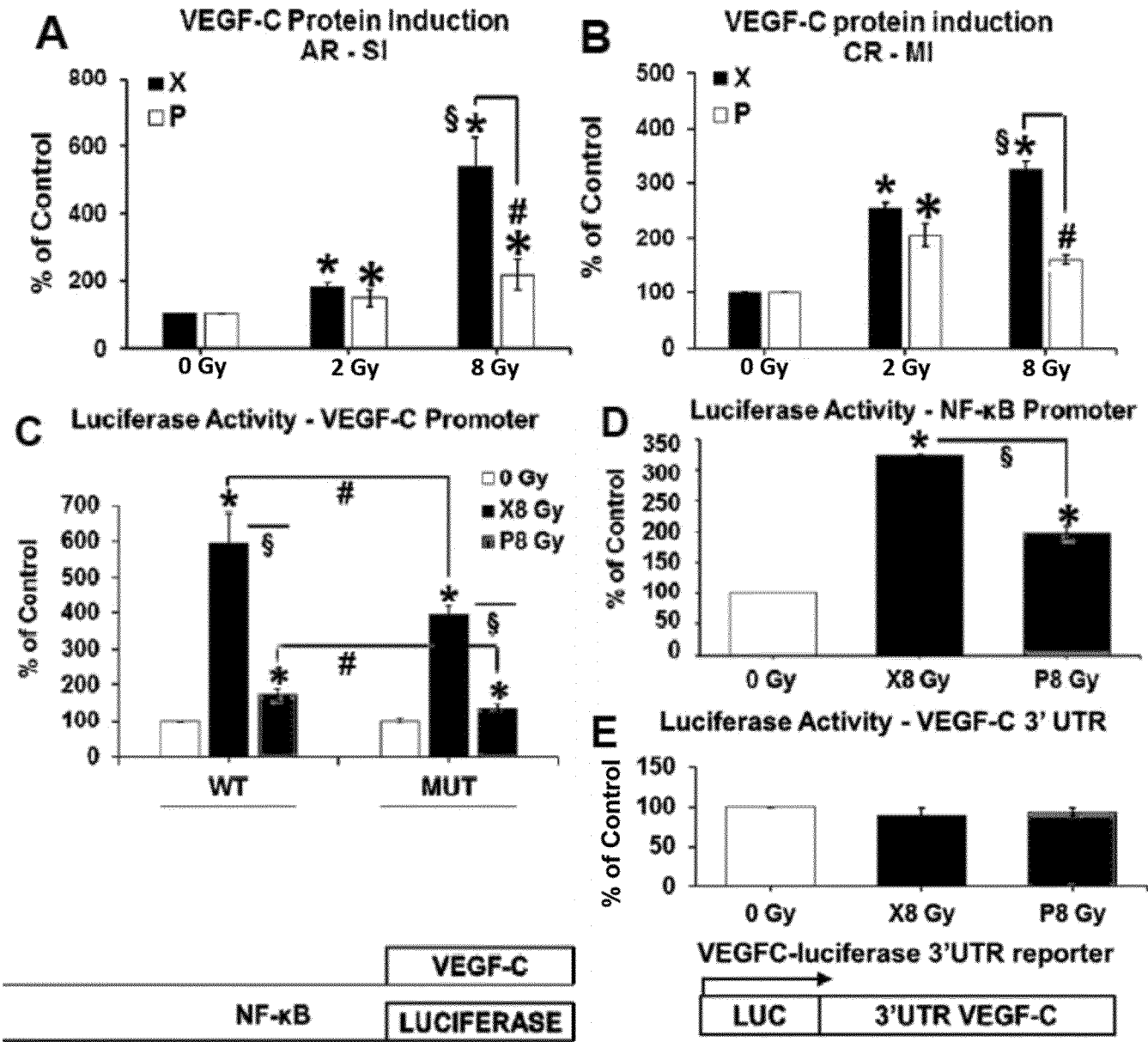


FIGURE 2

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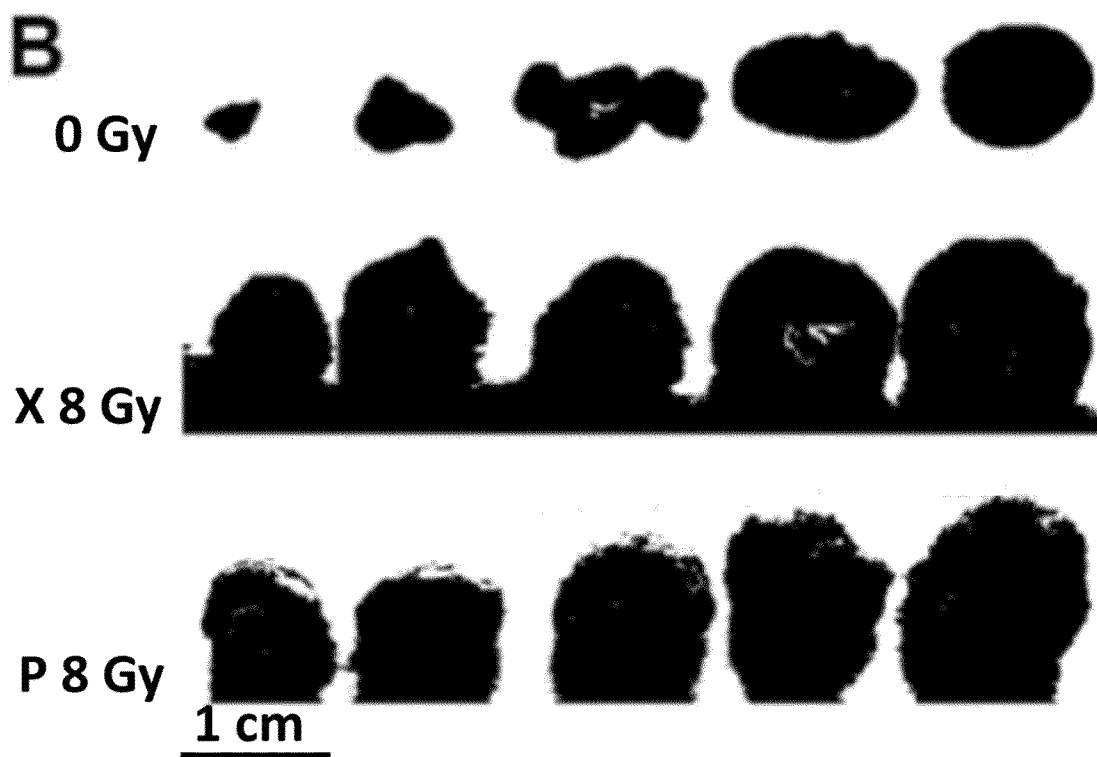
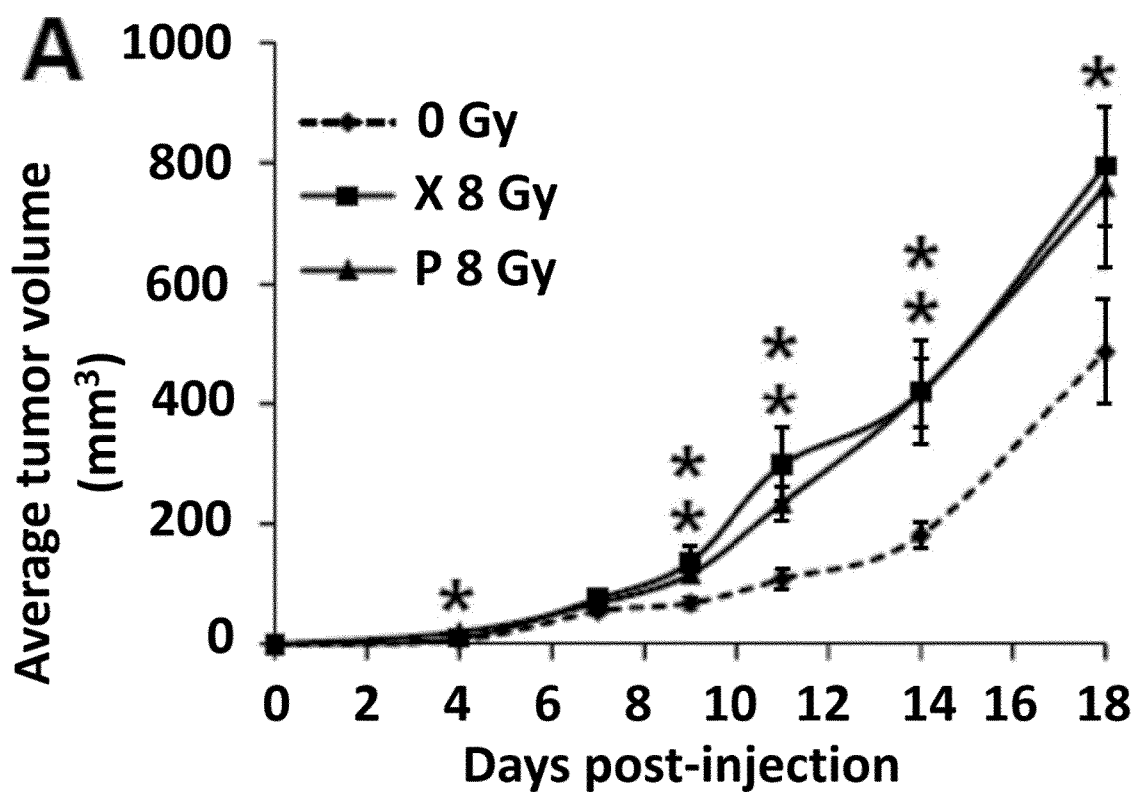


FIGURE 3

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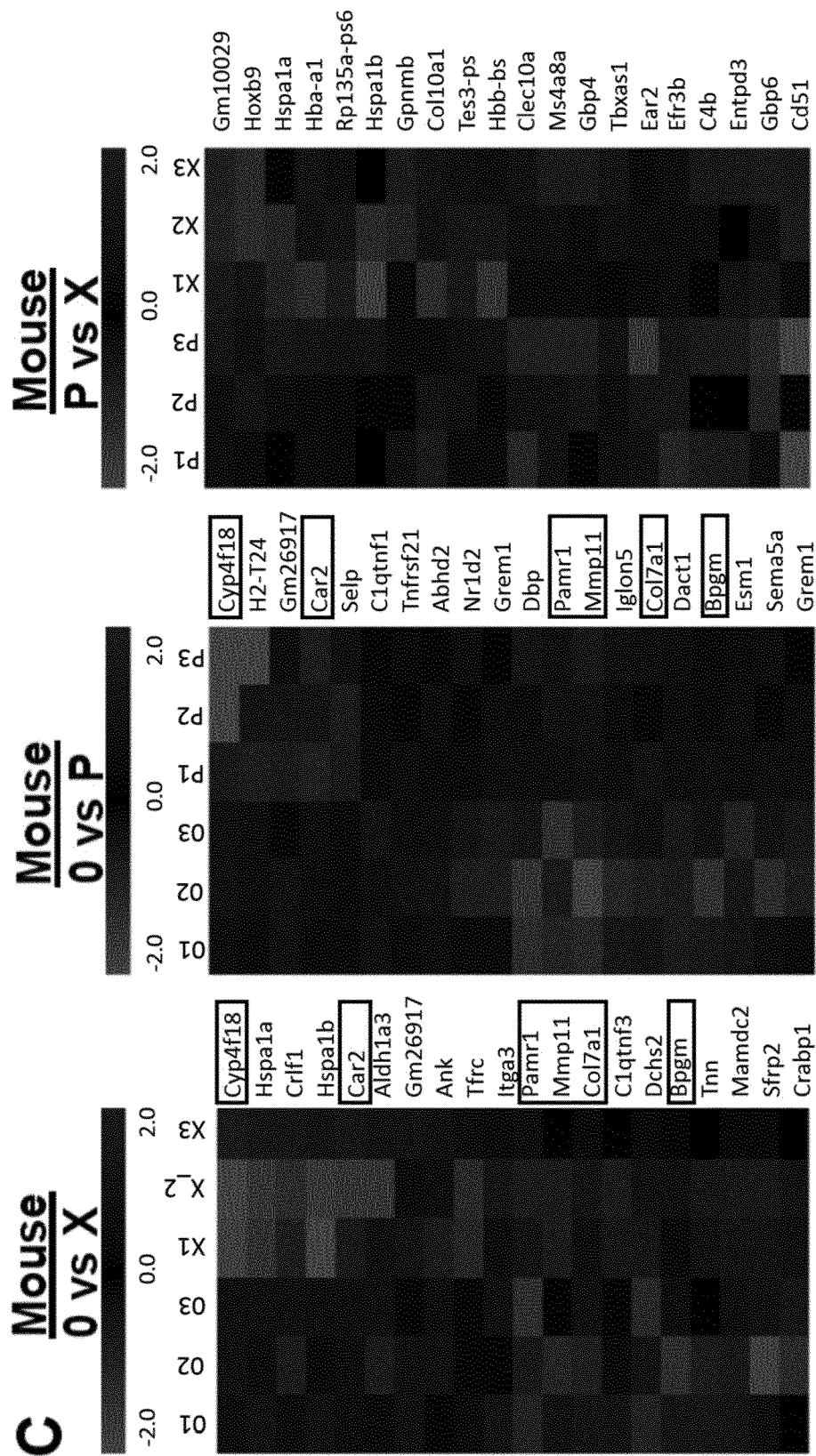


FIGURE 3 (Following)

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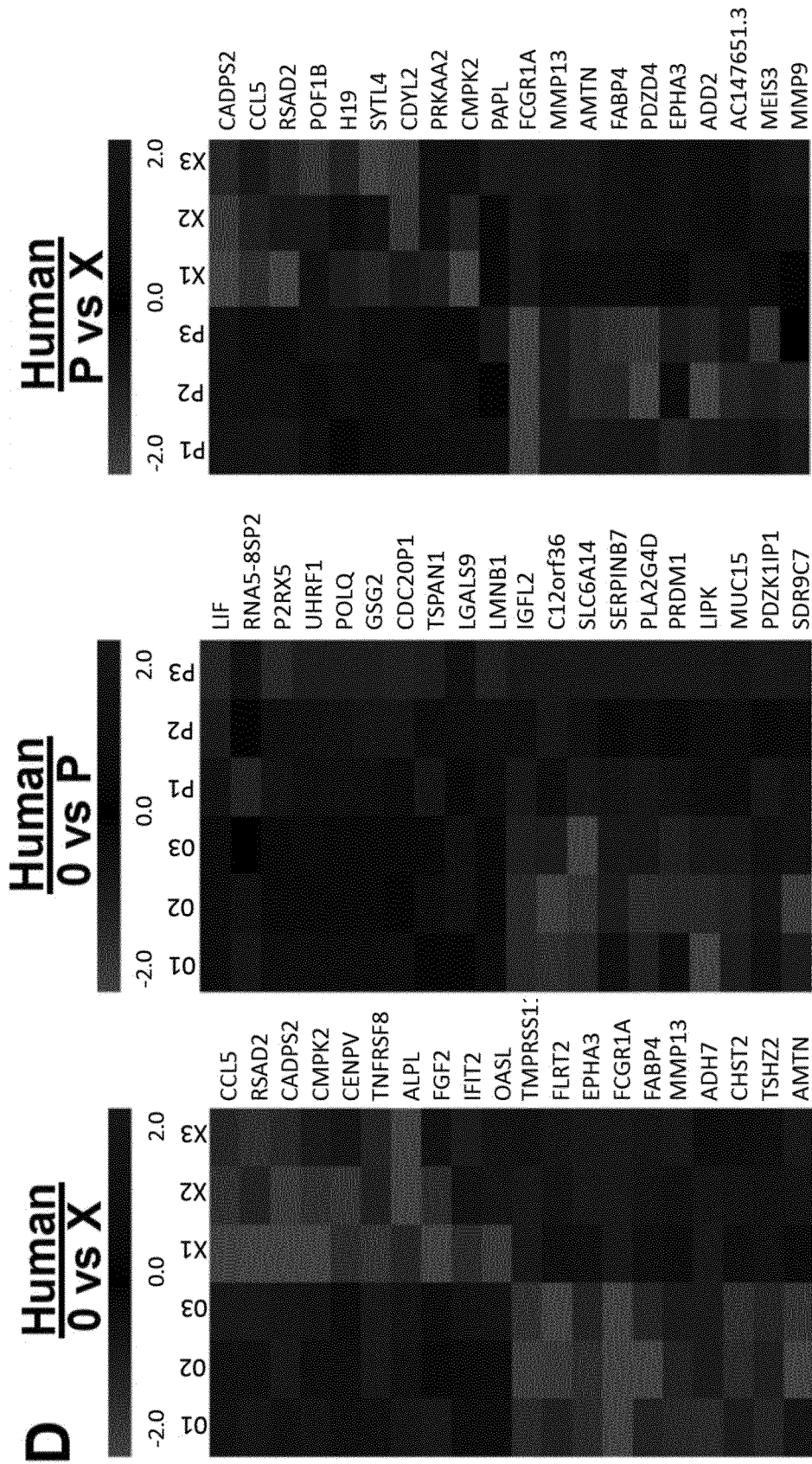


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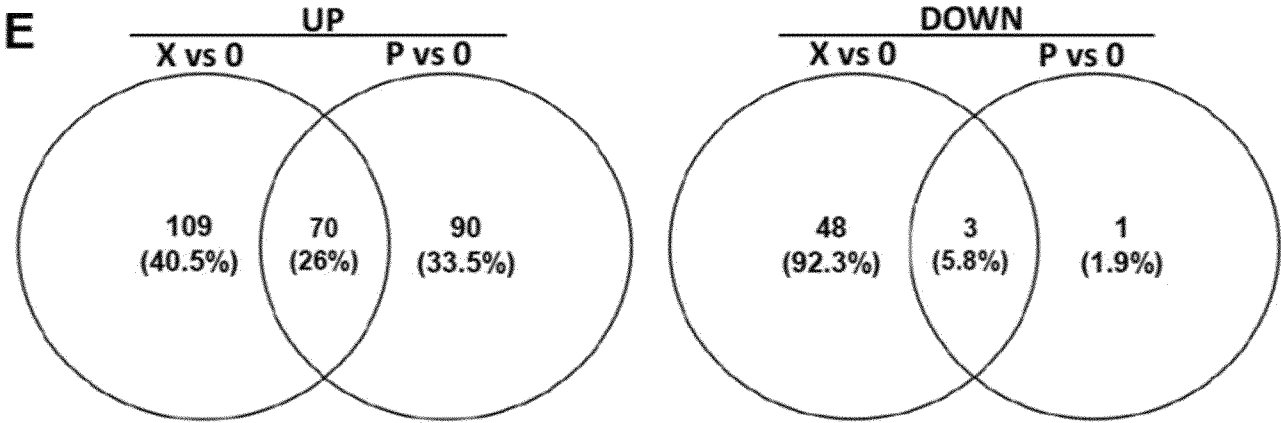


FIGURE 3 (Following)

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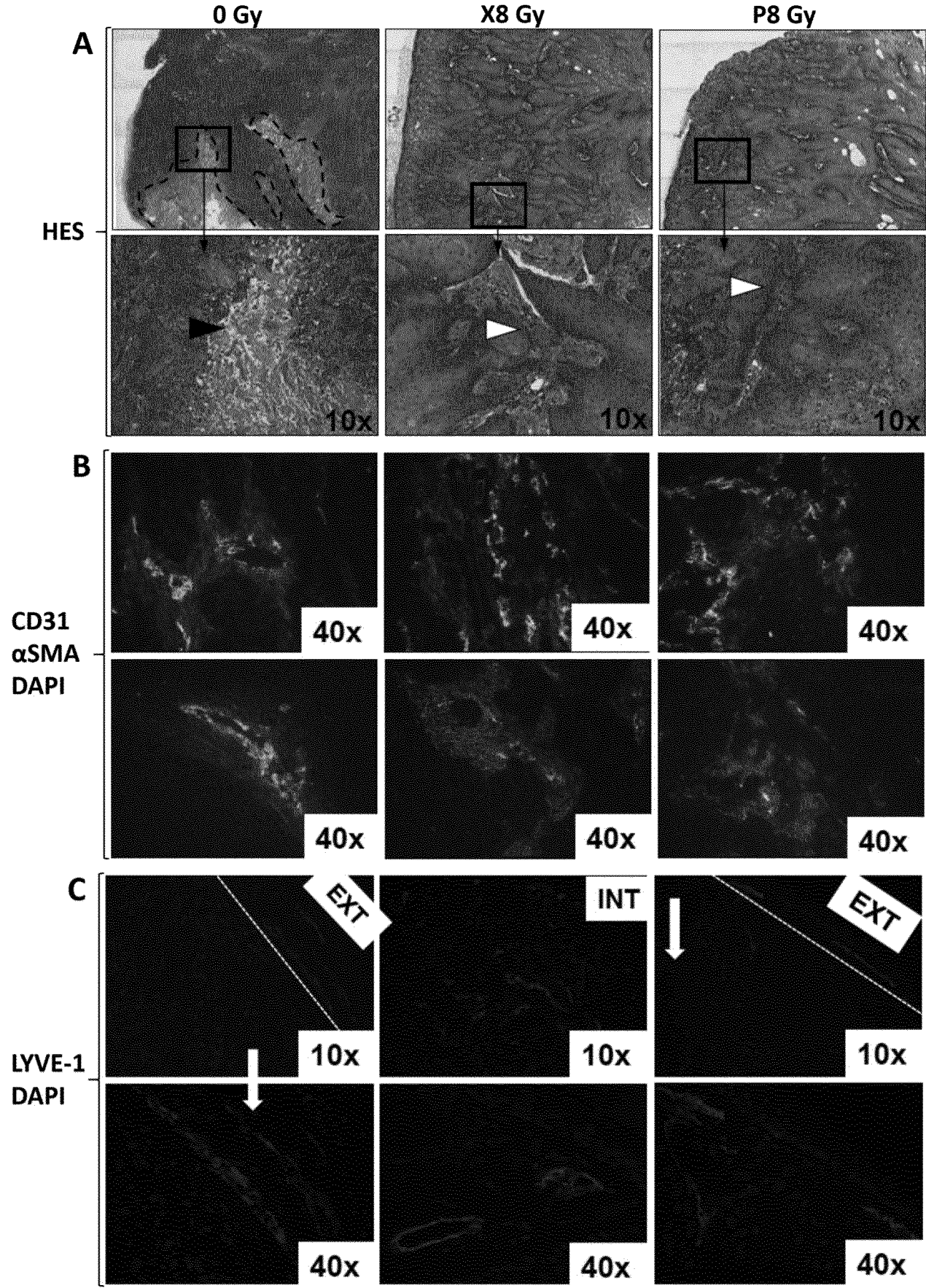


FIGURE 4

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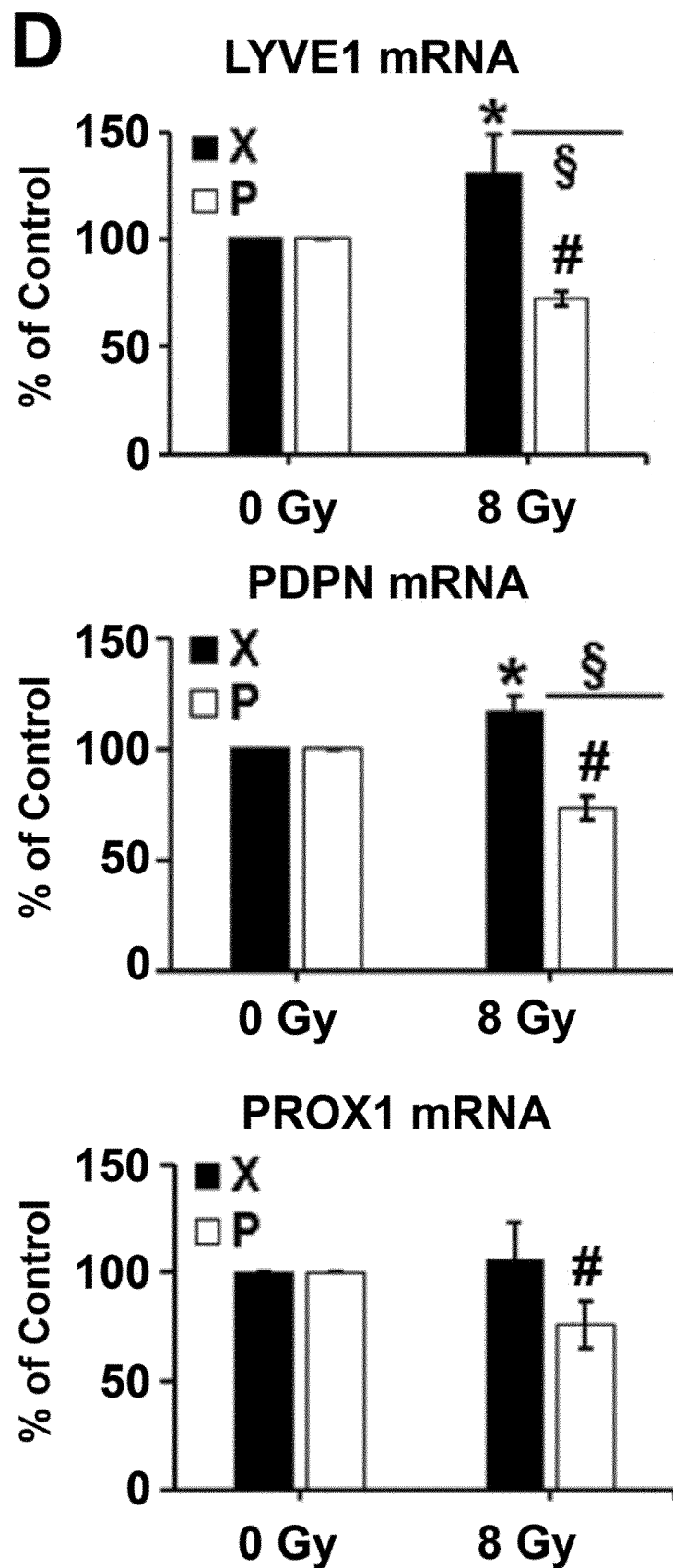


FIGURE 4 (Following)

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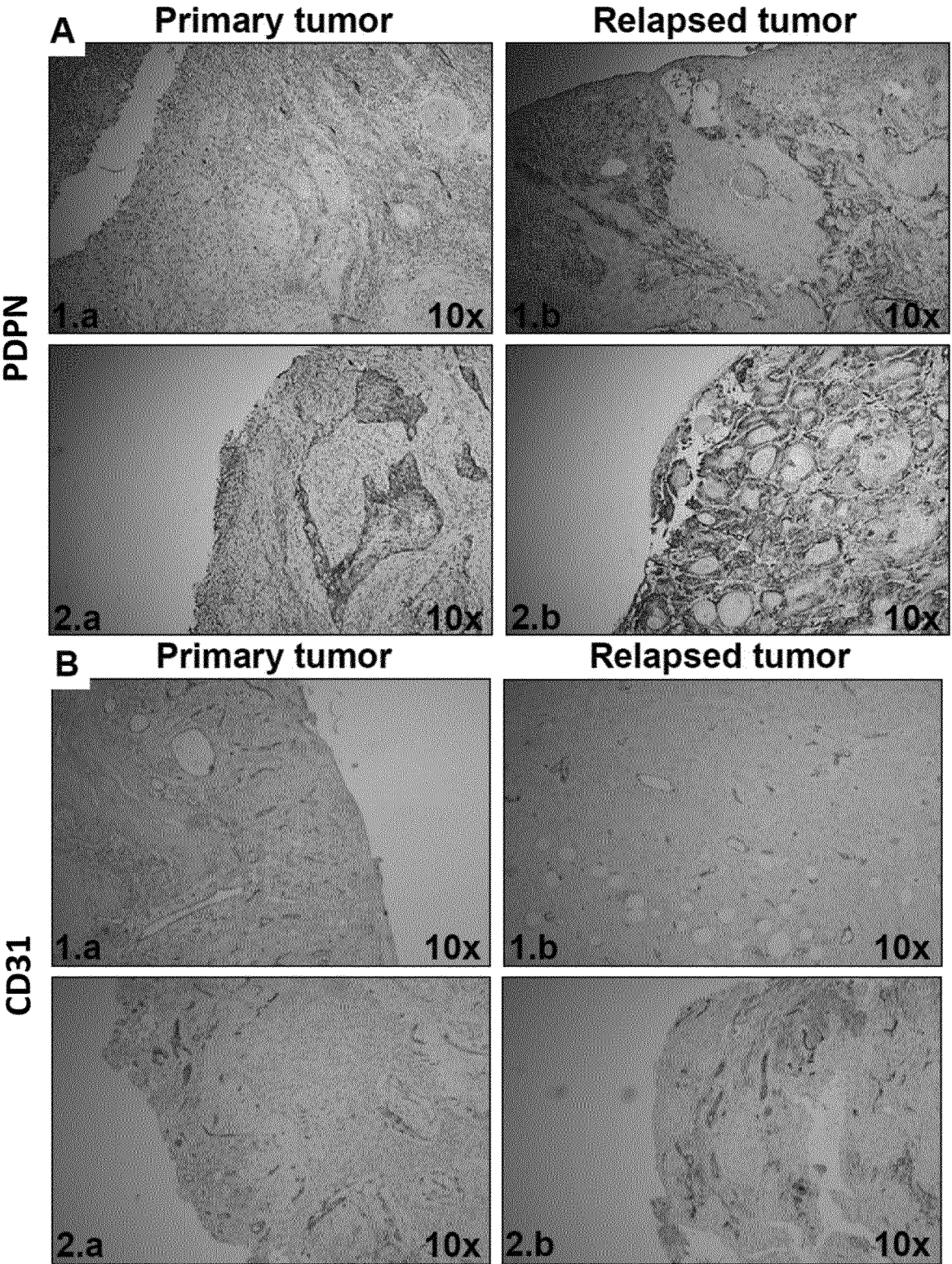


FIGURE 5

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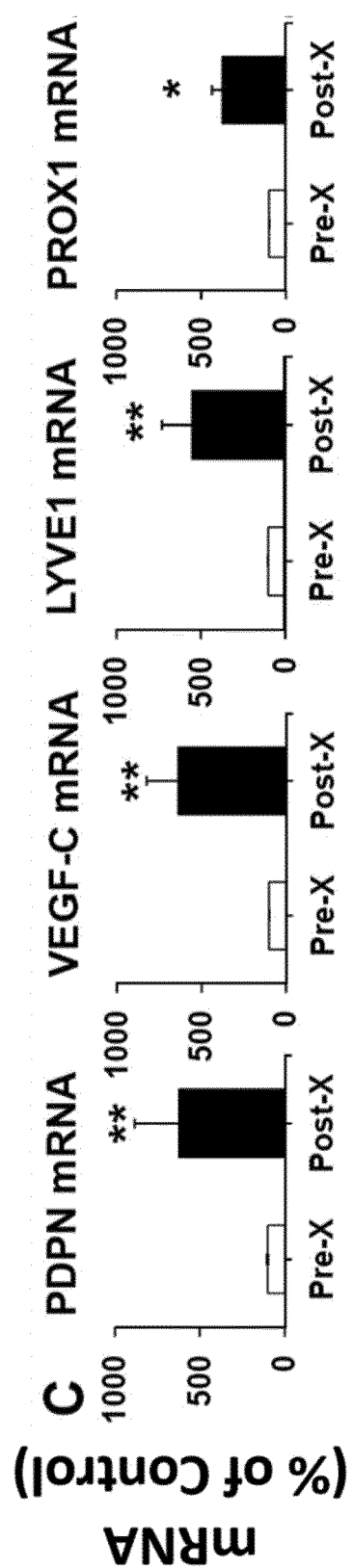
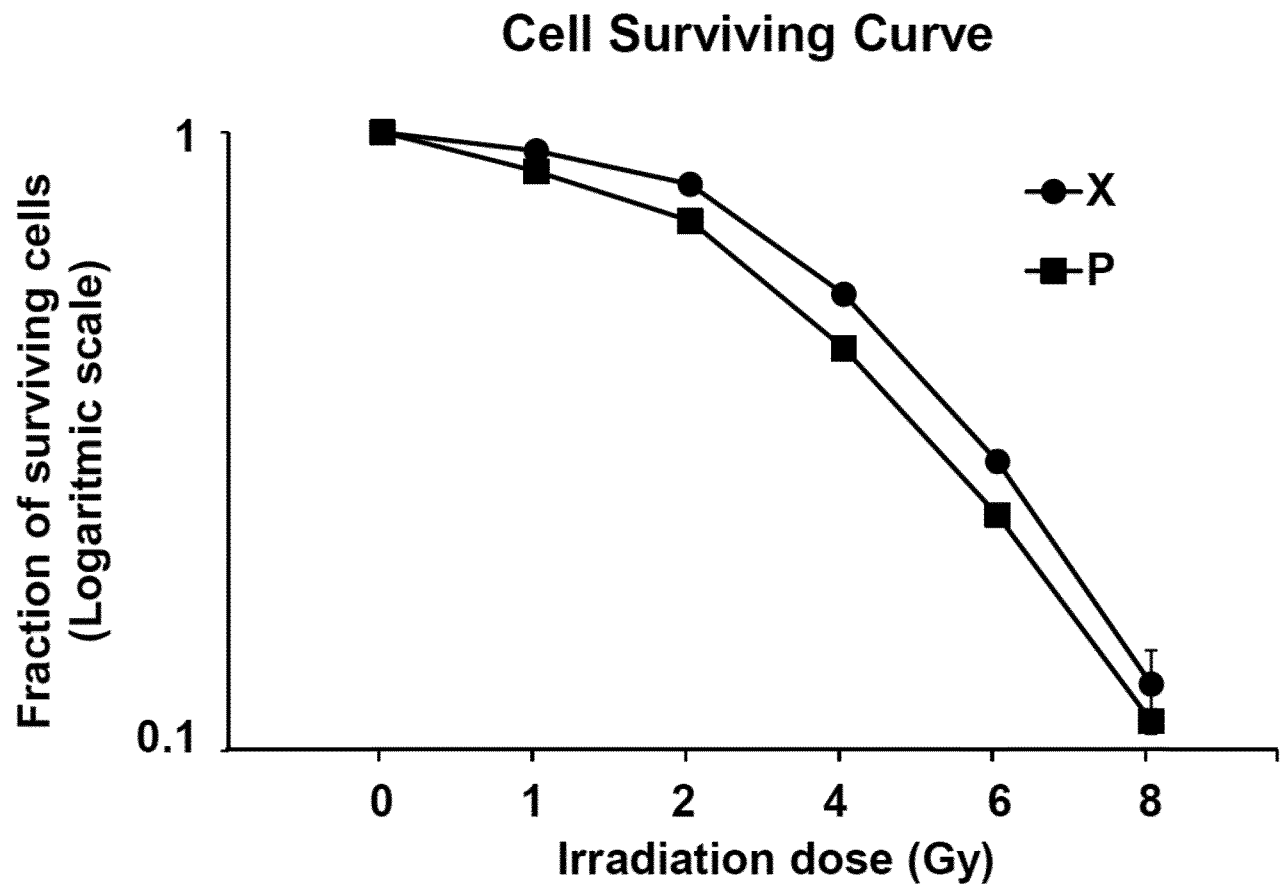


FIGURE 5 (Following)

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**FIGURE 6**

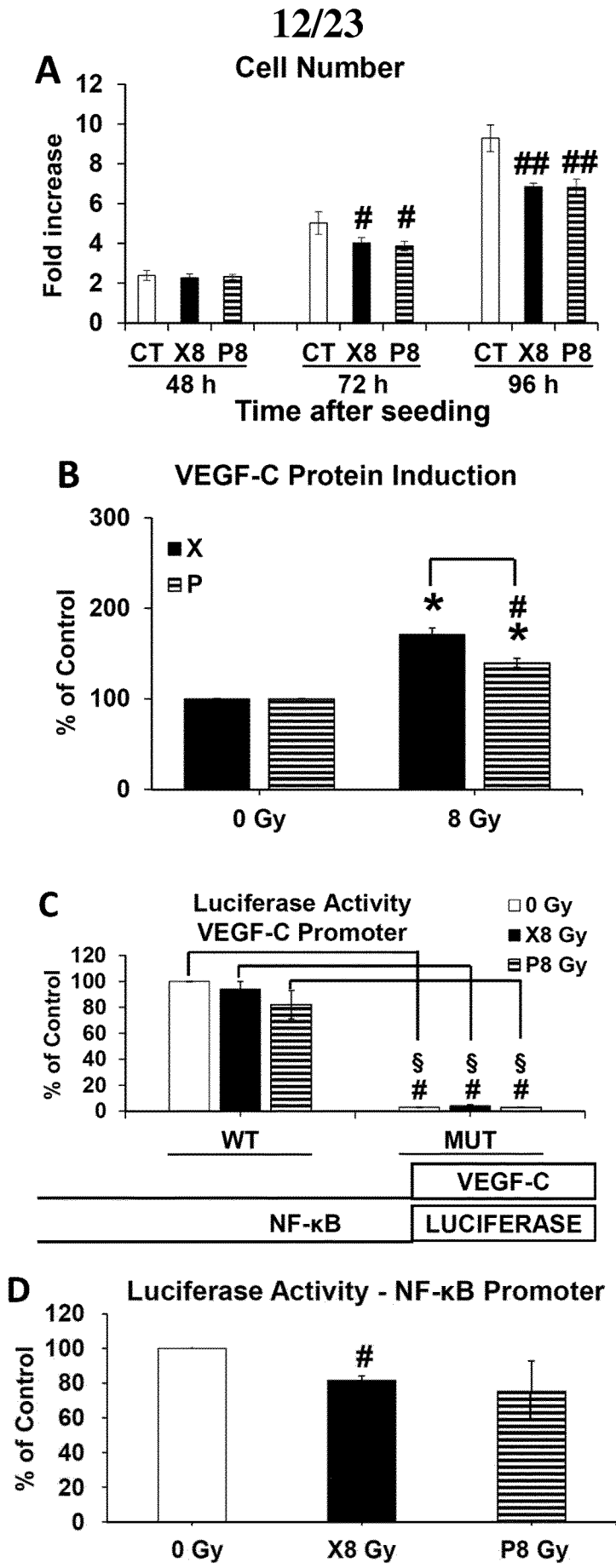


FIGURE 7

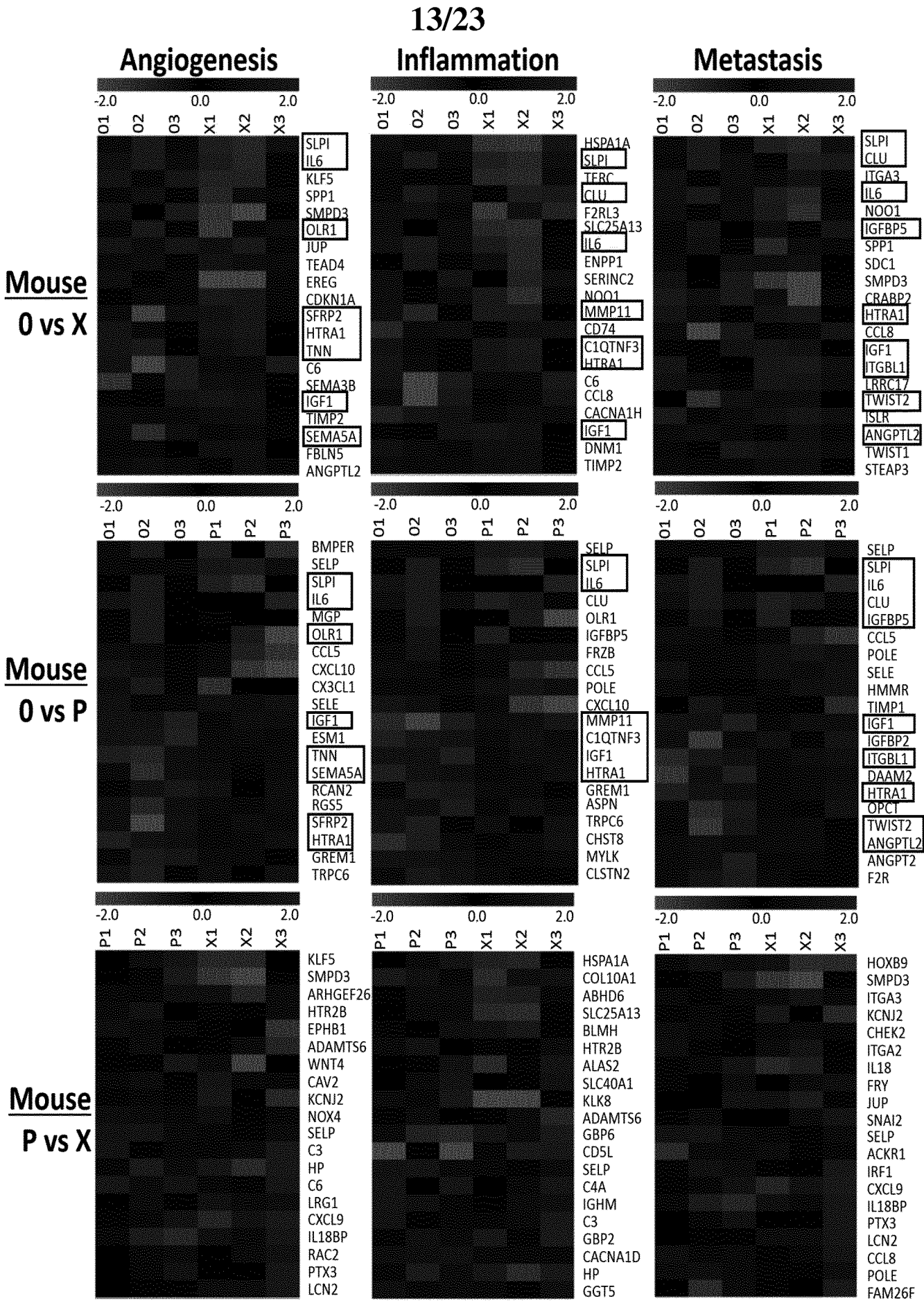


FIGURE 8

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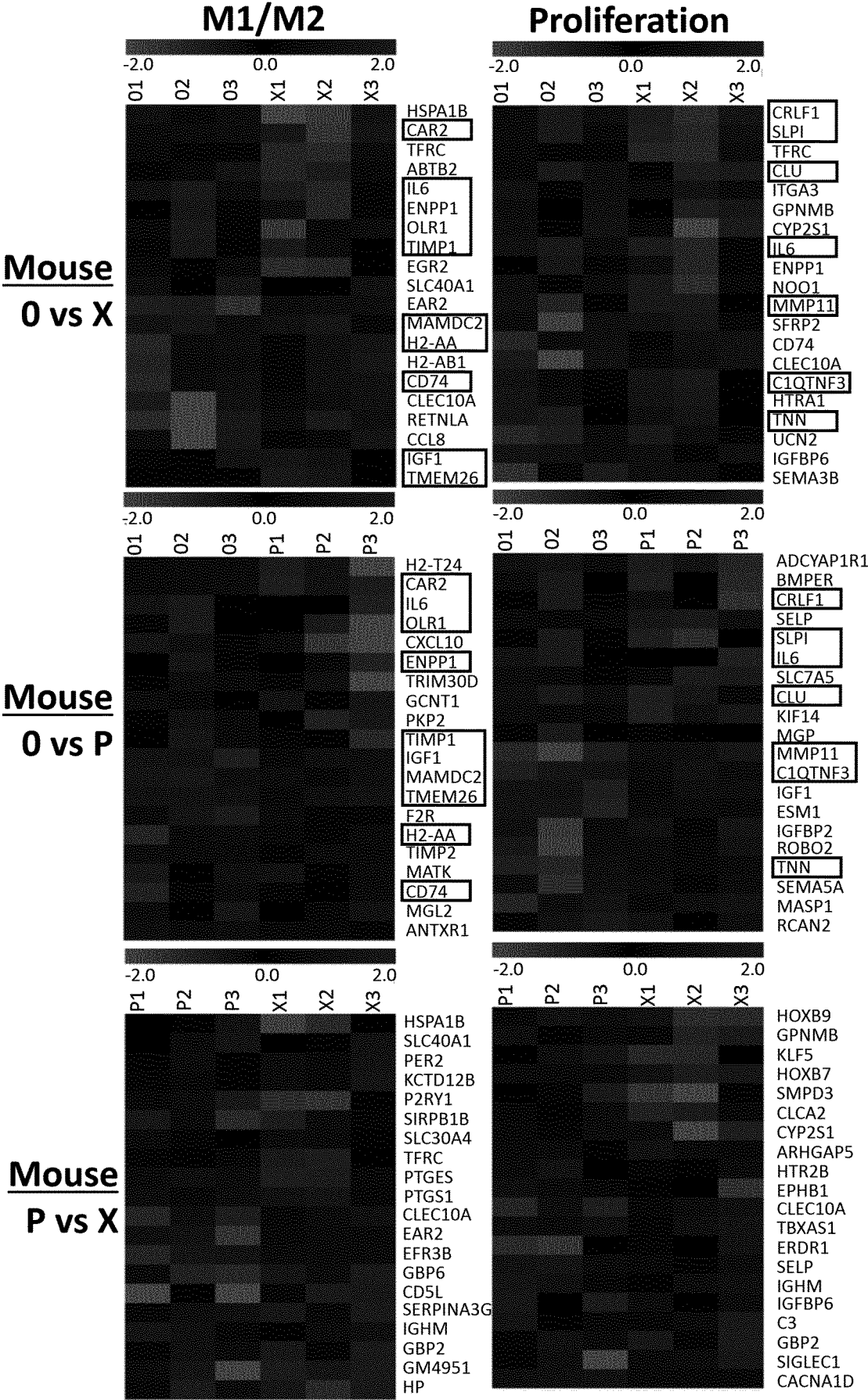


FIGURE 8 (Following)

Inflammation

Metastasis

Human
P vs X

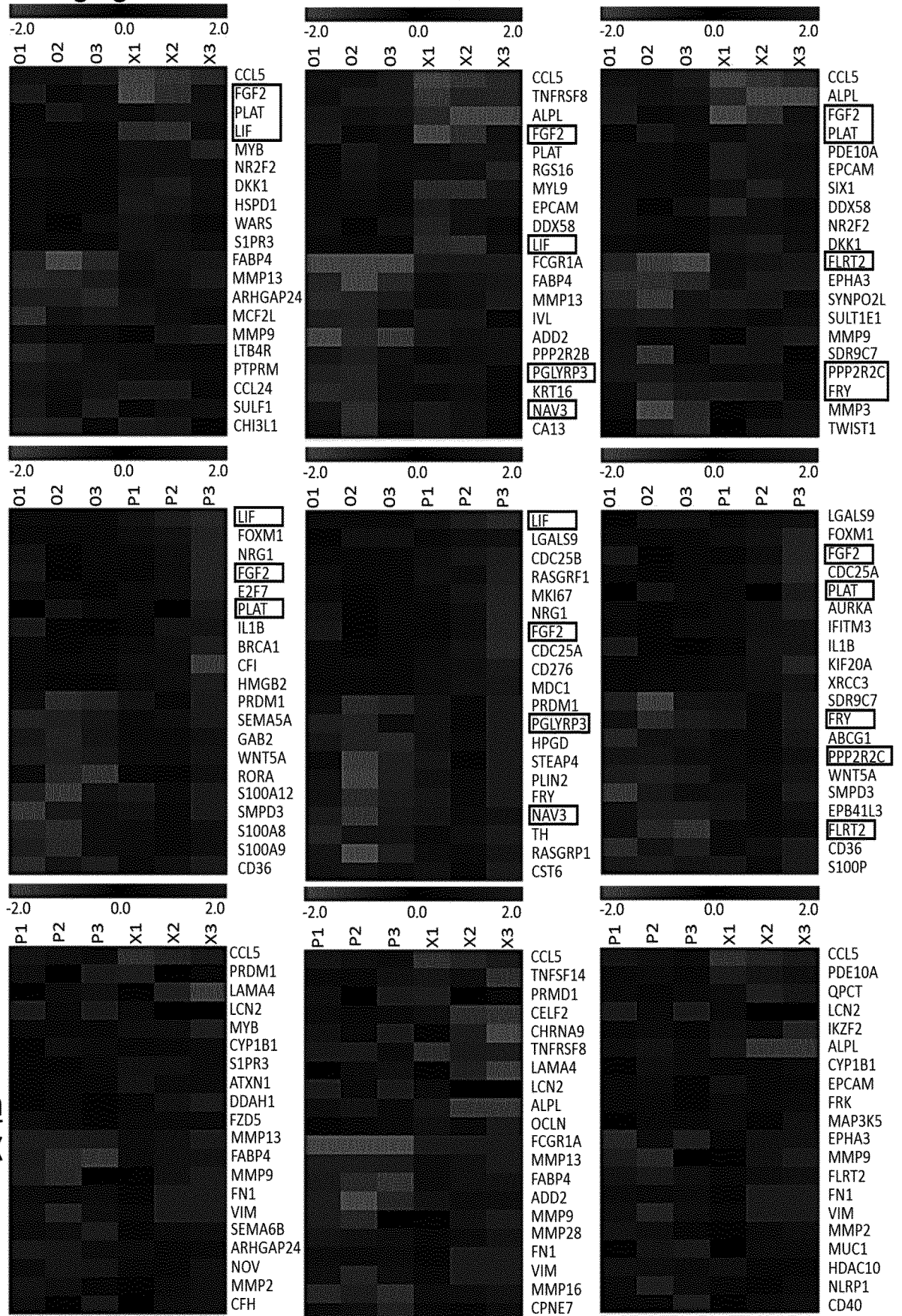


FIGURE 9

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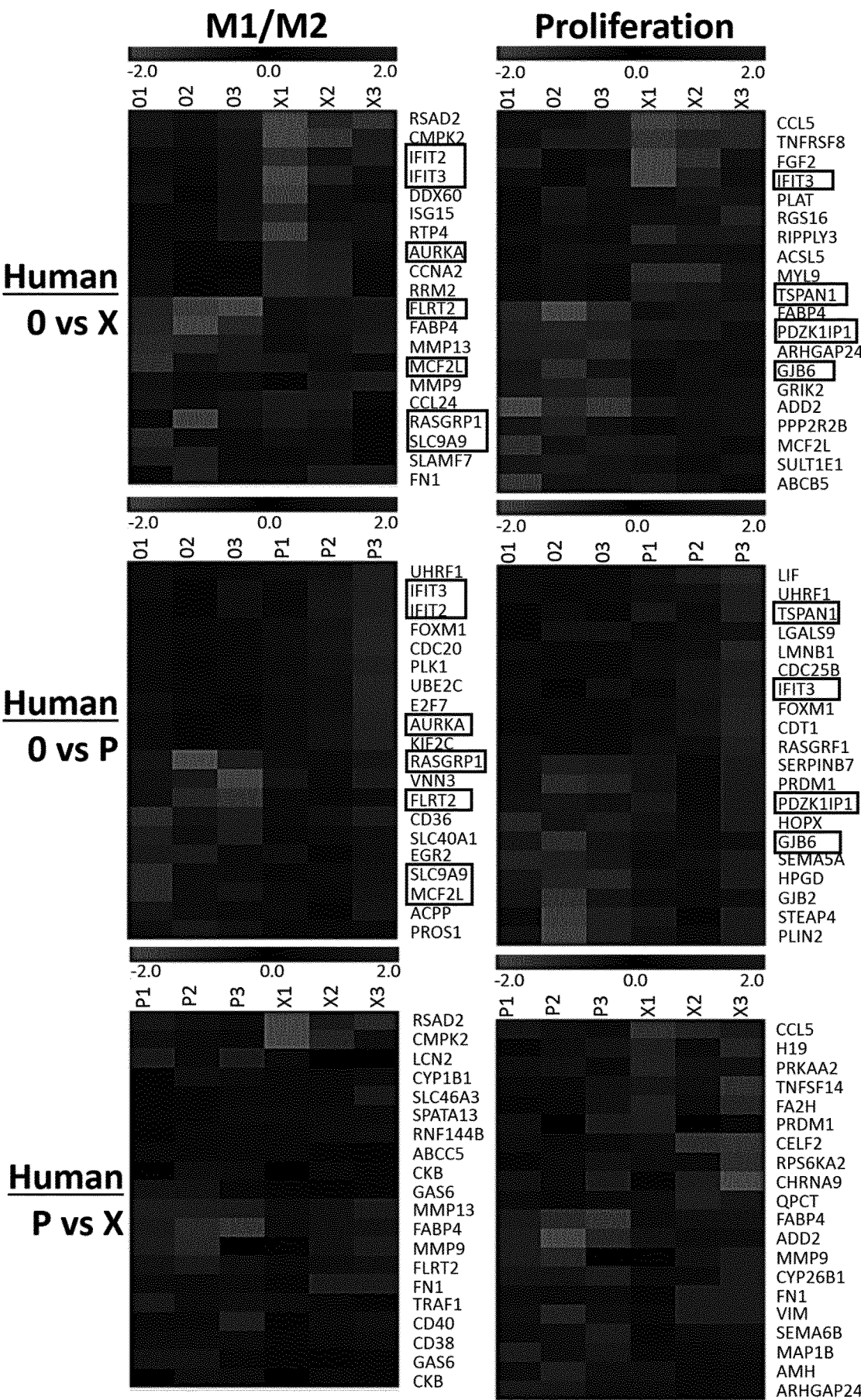


FIGURE 9 (Following)

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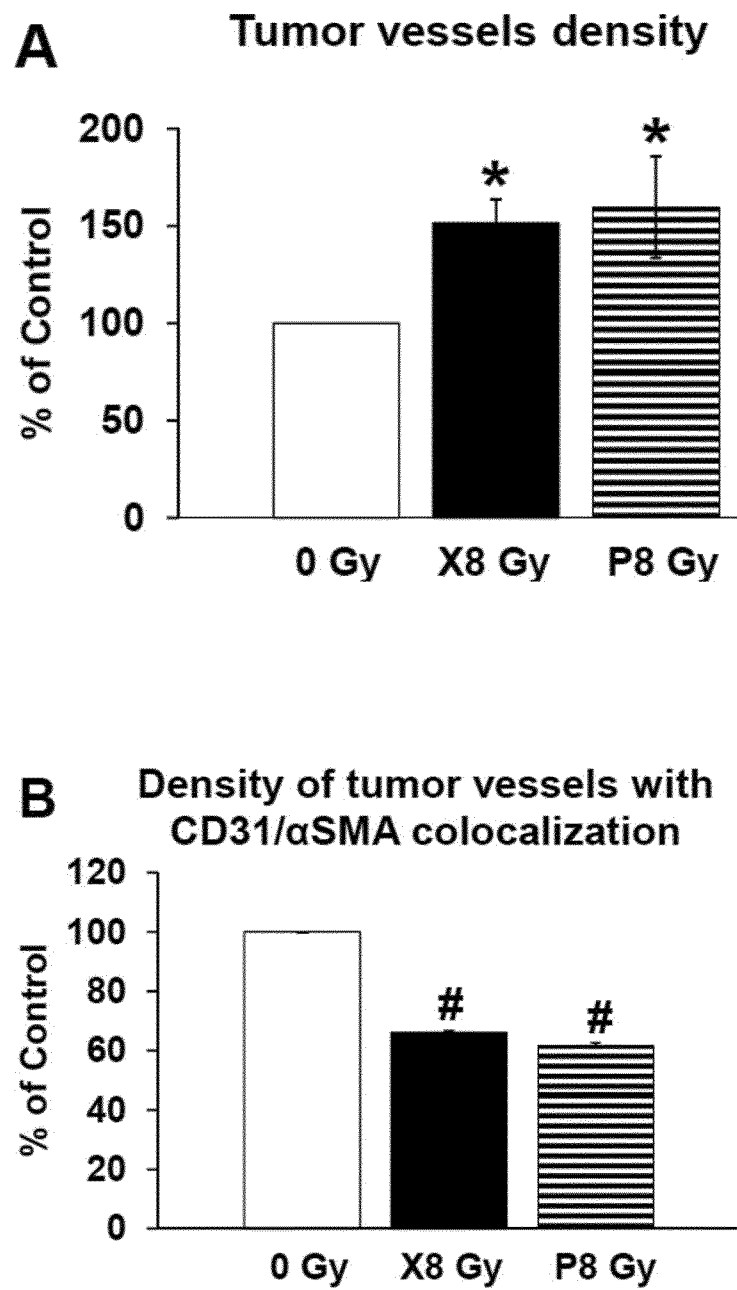


FIGURE 10

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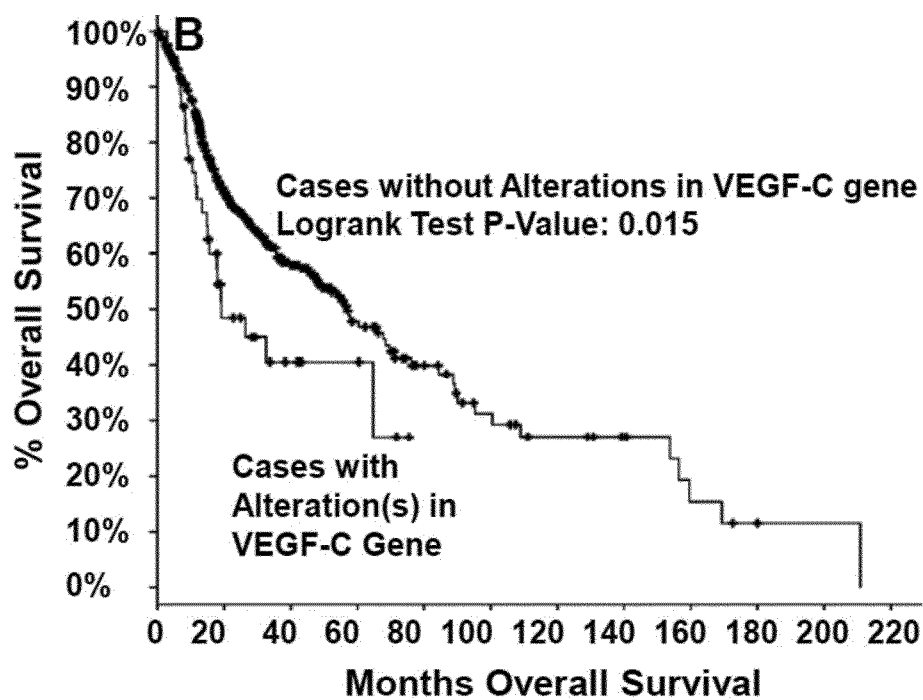
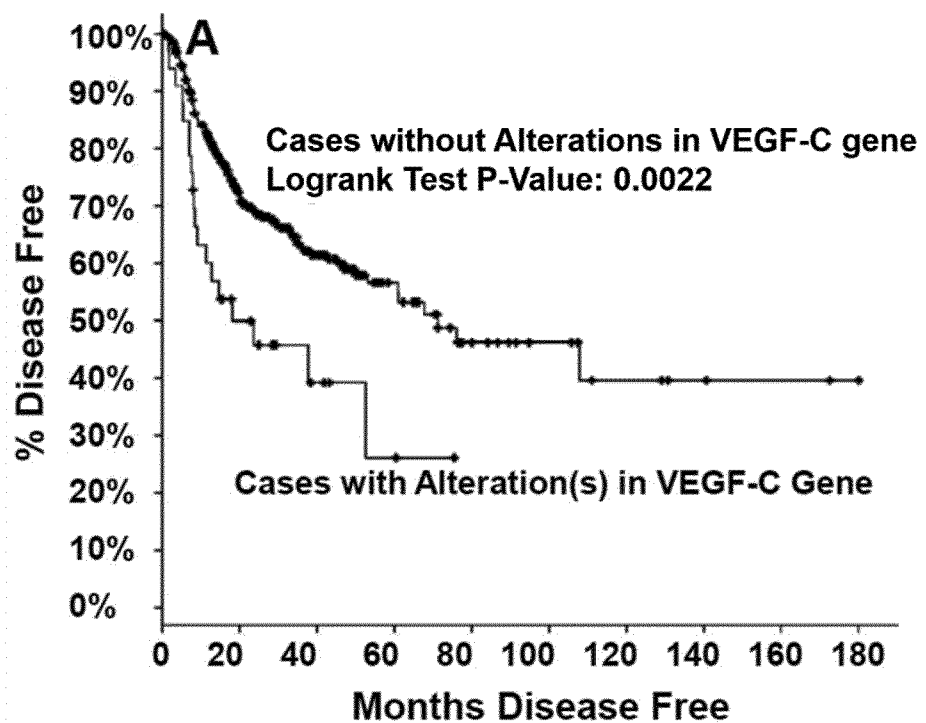
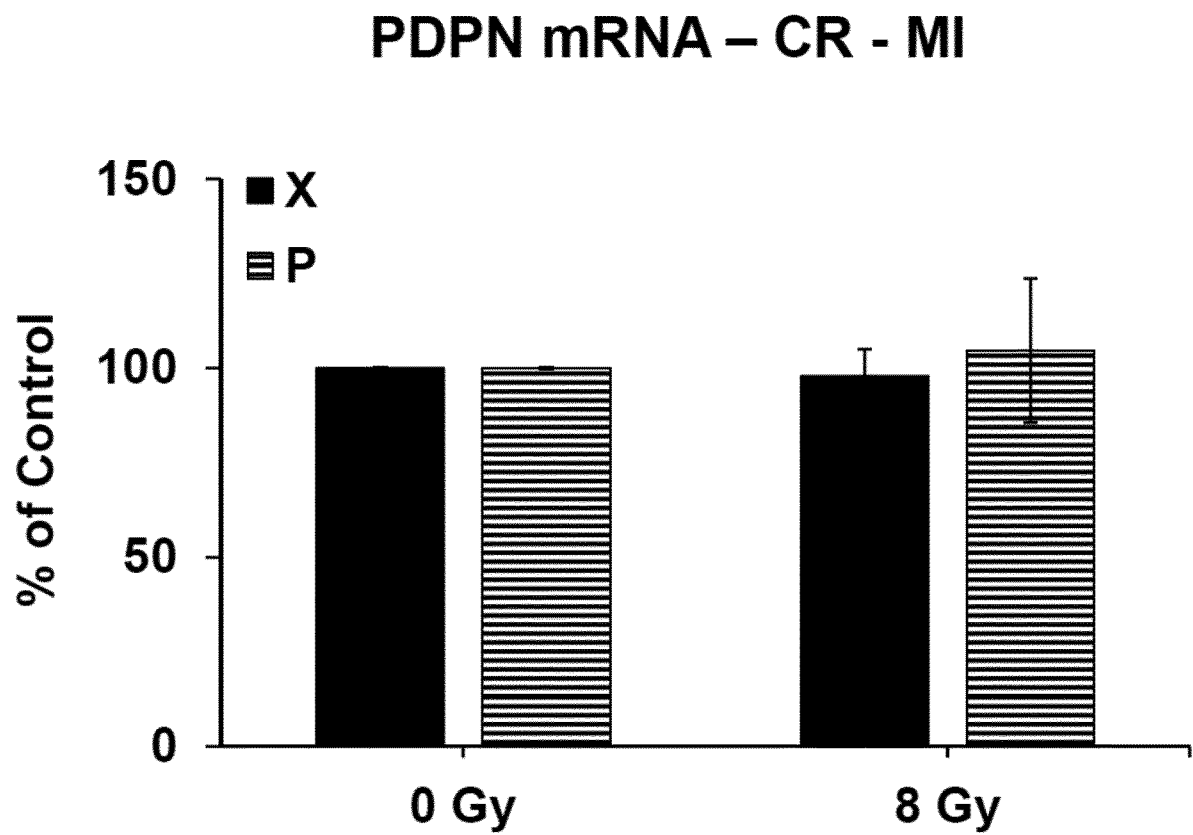


FIGURE 11

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**FIGURE 12**

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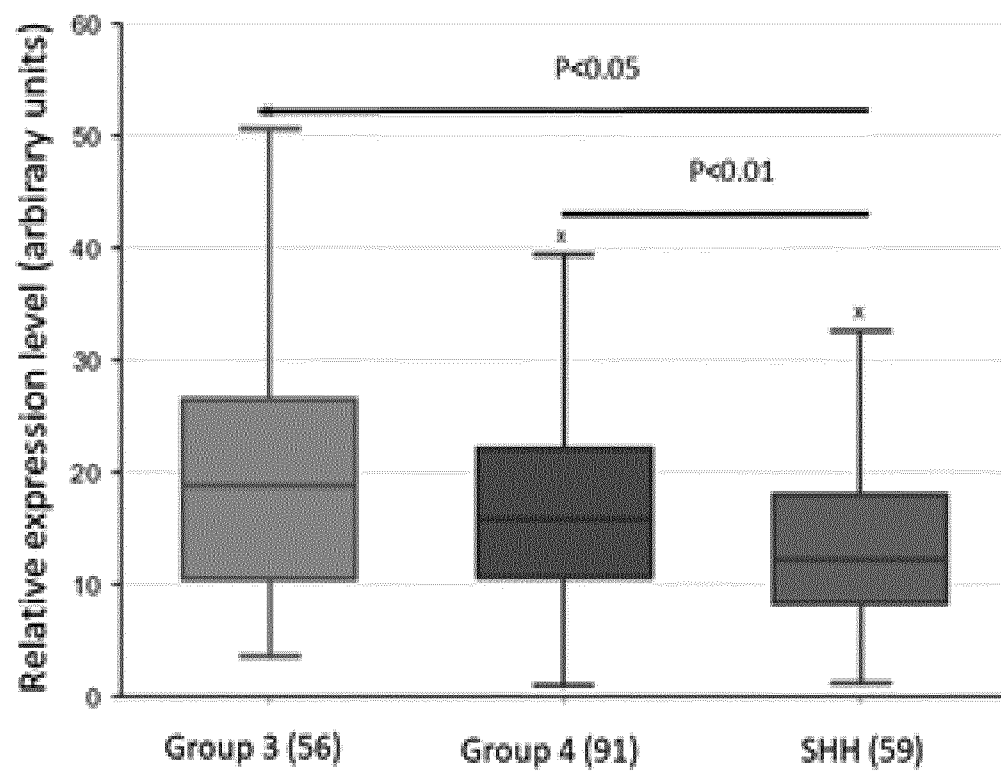


FIGURE 13

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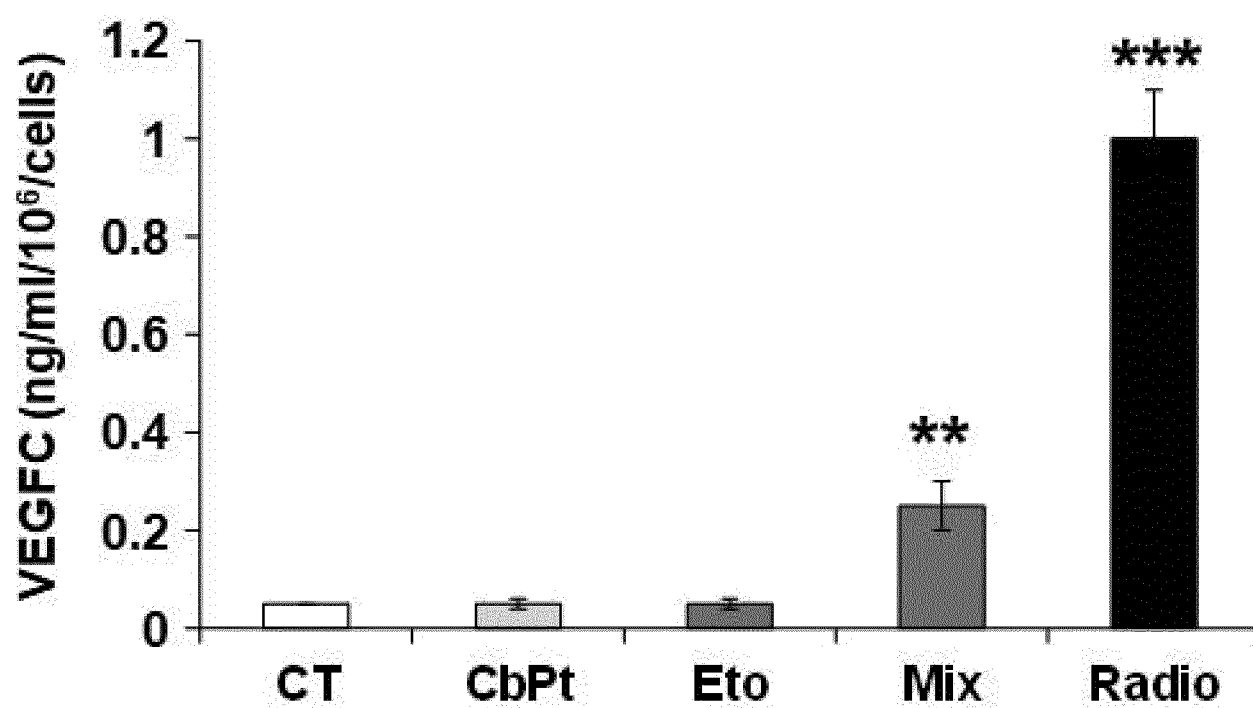


FIGURE 14

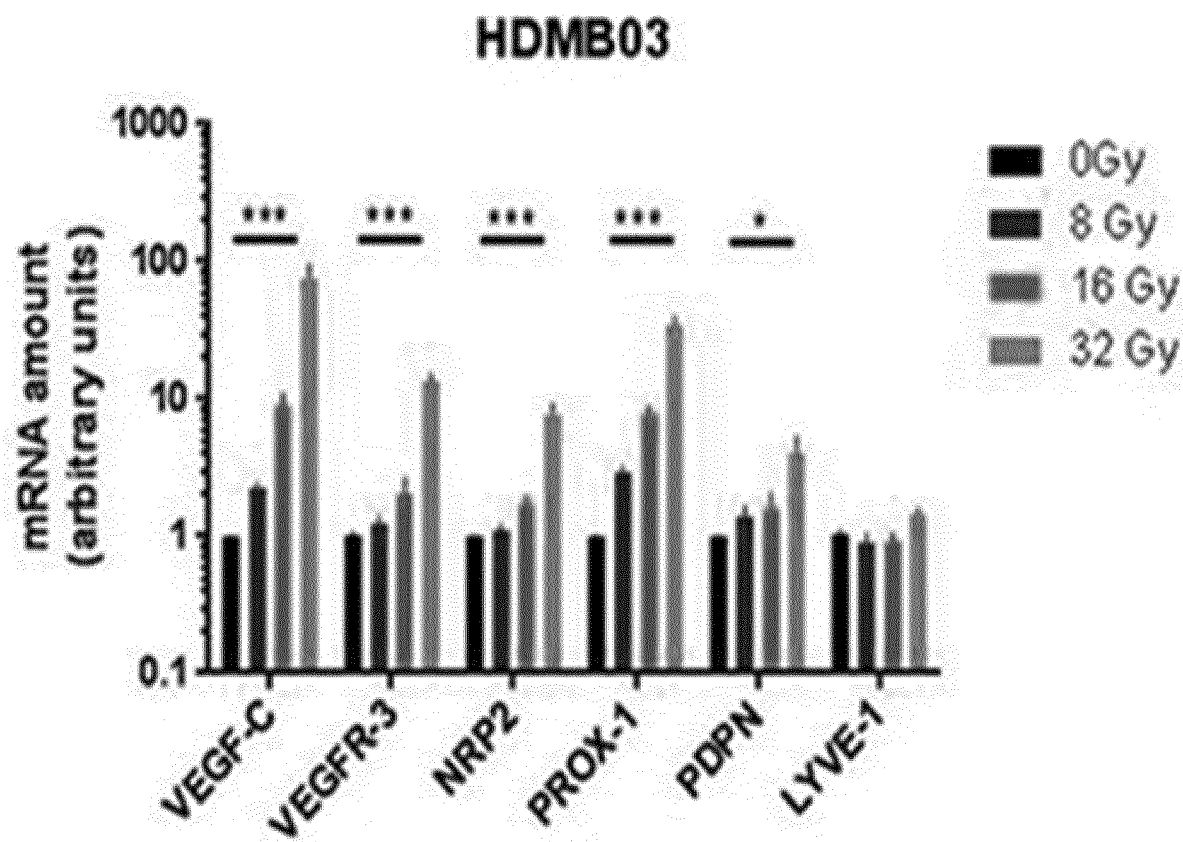


FIGURE 15

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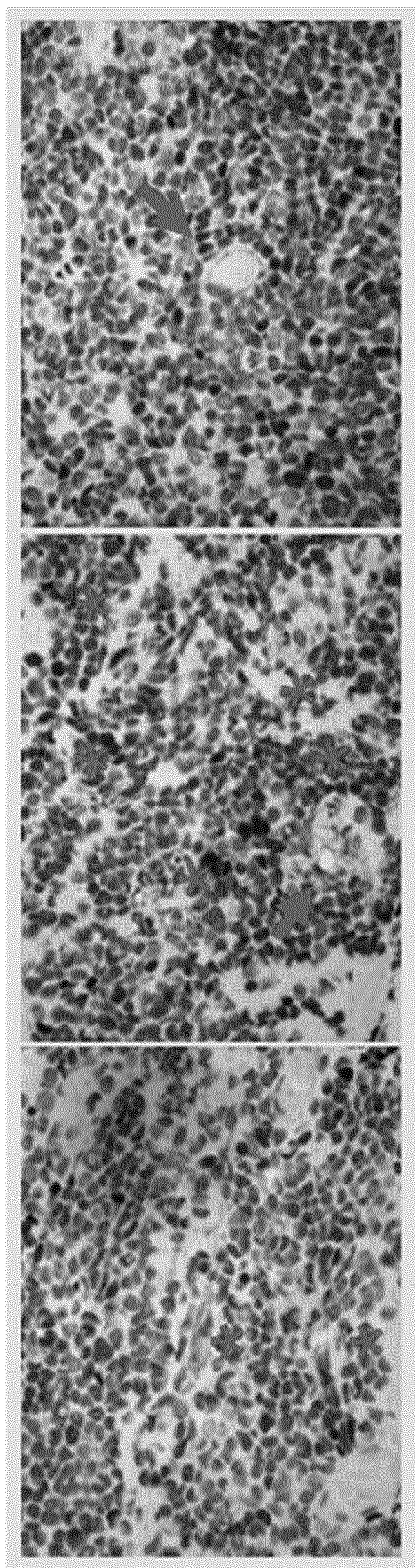


FIGURE 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/059613

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/053717 A1 (BEAUMONT HOSPITAL WILLIAM [US]; AKERVALL JAN [US]) 14 May 2010 (2010-05-14) the whole document	1-3,5
A	WO 2007/015935 A2 (BAYER HEALTHCARE LLC; WIRTZ RALPH MARKUS [DE]) 8 February 2007 (2007-02-08)	1-11
A	CN 104 894 229 A (UNIV CENTRAL SOUTH) 9 September 2015 (2015-09-09)	1-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

21 June 2018

Date of mailing of the international search report

27/06/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Cornelis, Karen

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/059613

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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