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(54) **Title**: METHOD FOR PREDICTING THE RESPONSIVENESS OF A PATIENT AFFECTED WITH AN OSTEOSARCOMA TO A CHEMOTHERAPY

(57) **Abstract**: The present invention relates to an ex vivo method for predicting the responsiveness of a patient affected with an osteosarcoma to chemotherapy, comprising the step of determining the level expression of at least one marker selected in the group consisting of β5 integrin, FAK or GSK-3β in a tumor sample from said patient. Moreover, the invention relate to an antagonist of β5 integrin, FAK or GSK-3β or an inhibitor of the expression of β5 integrin, FAK or GSK-3β for use in a method for enhancing clinical efficiency of chemotherapy.

METHOD FOR PREDICTING THE RESPONSIVENESS OF A PATIENT AFFECTED WITH AN OSTEOSARCOMA TO A CHEMOTHERAPY

FIELD OF THE INVENTION:

The present invention relates to an ex vivo method for predicting the responsiveness of a patient affected with an osteosarcoma to chemotherapy, comprising the step of determining the level expression of at least one marker selected in the group consisting of β5 integrin, FAK or GSK-3β in a tumor sample from said patient. Moreover, the invention relate to an antagonist of β5 integrin, FAK or GSK-3β or an inhibitor of the expression of β5 integrin, FAK or GSK-3β for use in a method for enhancing clinical efficiency of chemotherapy.

BACKGROUND OF THE INVENTION:

Primary osteosarcoma is the most common primary bone tumour that predominantly develops in adolescents and young adults. Survival has been improved, during the last two decades by the introduction of neoadjuvant chemotherapy, to induce necrosis of the tumour before surgery, combined with wide-margin, limb sparing surgery. Nevertheless, recurrent disease still occurs in about 30-40 % of patients and more than 70 % of them die of their tumour. The main prognostic factors are the presence of metastases and the response to chemotherapy evaluated by the percentage of tumoral necrosis of the surgical resection [Meyers et al, 1992 and Bacci et al, 1997]. A more intensified therapy didn't give a survival benefice in poor responders, suggesting that there may be an intrinsic biological difference between good and poor responsive tumours to chemotherapy.

Few studies have been conducted to identify new factors able to predict the response of the tumour to chemotherapy, the tumour aggressiveness and its ability to develop metastases. Recently, the protein Ezrin, member of the ERM (Ezrin-Radixin-Moesin) family which is a membrane cytoskeletal linker protein which is produced by the expression of the Vil2 gene has been identified as a potential independent negative prognostic marker for event-free survival and overall survival in high grade osteosarcoma [Salas et al, 2007 and Kim et al, 2009]. It has been also demonstrated that the mTOR/p70S6K signal transduction pathway contributes to OS progression and patient's prognosis. Recent results have also shown that HSD17B10 gene expression, a mitochondrial enzyme implicated in the production of

dihydrotestosterone (DHT), is up-regulated in poor responders and that immunohistochemistry expression of HSD17B10 on biopsy before treatment is correlated to response to chemotherapy [Salas et al, 2009].

Despite these results, there is a permanent need in the art for new markers to differentiate good and poor responsive tumours to chemotherapy.

Cell adhesion to extracellular matrix is known to confer a cell-adhesion-mediated chemotherapeutic drug resistance for solid tumour [Damiano et al, 2002]. Tumour adhesion and invasiveness are in part mediated by the interaction of the tumour cells with extracellular matrix, through a family of membrane receptors, the integrins. All integrins are structurally similar heterodimers composed of α- and β-subunit transmembrane glycoproteins that are non-covalently linked. Integrins play pivotal roles in diverse cellular processes, such as migration, proliferation and attachement, angiogenesis and survival. Ligand binding to integrins leads to integrin clustering and to the association with proteins as FAK (focal adhesion kinase) or ILK (integrin-linked kinase) in focal adhesion clusters and recruitment of actin filaments regulated by the small GTPase RhoB thus transducing signalling across the membrane [Hood et al, 2002]. Several types of cancer cells (breast cancer, prostate cancer, colon cancer, lung carcinoma, uterin cervix carcinoma and glioma), express β3 integrin; such expression has been shown to be associated with progression and metastasis in breast and prostate cancer [Felding-Habermann et al, 2001 and Hullinger et al, 1998]. Few studies have underlined the role of β3 and β5 integrins in cell response to a genotoxic stress. The inventors have demonstrated that β3 and β5 integrin control through ILK and RhoB the radioresistance of glioblastoma cell lines through the modulation of the mitotic cell death [Monferran et al, 2008] and that the co-expression of integrin β3 and FGF-2 in the biopsy of patients treated with exclusive radio-chemotherapy for locally advanced non small lung carcinoma was predictive of local control after treatment [Massabeau et al, 2009]. β5 has been shown to be involved in the resistance of ovarian cancer to cisplatin [Maubant et al, 2002] and the β3 integrin to be involved in the resistance to taxol of human breast cancer MCF7 cell line [Menendez et al, 2005].

The inventors have also recently demonstrated that the β3 and β5 integrin pathway was also largely involved in the control of hypoxia, which plays a crucial role in tumour aggressiveness and resistance to chemotherapy and radiotherapy. Indeed, they have shown that hypoxia activates RhoB and that β3 and β5 integrins control RhoB activation under hypoxic conditions through FAK, then controlling HIF-1α expression via GSK-3β [Skuli et

al, 2009]. Moreover, inhibiting RhoB but also β3 integrin or FAK in glioblastoma xenografts led to a significant tumor oxygenation and vascularization normalization in part due to inhibition of MMP2 and Ang-2 expression [Skuli et al, 2009 and Ader et al, 2003]} Few other studies have described the involvement of integrin in hypoxia pathways The induction of integrin's expression by hypoxia has been shown in breast cancer cells for β4 integrin [Yoon et al, 2006] and in melanoma cells for β3 integrins.

The inventors hypothesized that the biological pathway that they demonstrated to be involved in survival after radiotherapy and in tumor hypoxia could be predictive of response to pre-operative chemotherapy and to clinical outcome on pre-treatment tumor biopsies of patients treated for a high grade osteosarcoma. They show for the first time that the β5 integrins hypoxia pathway through FAK and GSK-3β is expressed in osteosarcoma and is implicated in the response to treatment. The results obtained by the inventors will open new therapeutic perspectives by using specific molecular therapies directed against this pathway.

SUMMARY OF THE INVENTION:

Thus, the invention relates to an ex vivo method for predicting the responsiveness of a patient affected with an osteosarcoma to chemotherapy, comprising the step of determining the level expression of at least one marker selected in the group consisting of β5 integrin, FAK or GSK-3β in a tumor sample from said patient.

Moreover, the invention relate to an antagonist of β5 integrin, FAK or GSK-3β or an inhibitor of the expression of β5 integrin, FAK or GSK-3β for use in a method for enhancing clinical efficiency of chemotherapy.

DETAILED DESCRIPTION OF THE INVENTION:

Definitions:

Throughout the specification, several terms are employed and are defined in the following paragraphs.

As used herein, the term "β5 integrin" has its general meaning in the art and refers to a member of the integrin's family which are receptors that mediate attachment between a cell and the tissues surrounding it, which may be other cells or the extracellular matrix (ECM).

They also play a role in cell signaling and thereby regulate cellular shape, motility, and the cell cycle.

As used herein, the term “FAK” or “Focal Adhesion Kinase” has its general meaning in the art and denotes a protein that, in humans, is encoded by the PTK2 gene. FAK is a focal adhesion-associated protein kinase involved in cellular adhesion and spreading processes.

As used herein, the term “GSK-3β” or “Glycogen synthase kinase 3 beta” has its general meaning in the art and denotes a proline-directed serine-threonine kinase that was initially identified as a phosphorylating and inactivating glycogen synthase. Two isoforms, alpha (GSK3A) and beta, show a high degree of amino acid homology. GSK3B is involved in energy metabolism, neuronal cell development, and body pattern formation.

As used herein, the term "patient", is intended for a human affected or likely to be affected with a tumor.

Method for predicting the responsiveness of a patient

In a first aspect, the invention relates to an ex vivo method for predicting the responsiveness of a patient affected with an osteosarcoma to chemotherapy, comprising the step of determining the level expression of at least one marker selected in the group consisting of β5 integrin, FAK or GSK-3β in a tumor sample from said patient.

In preferred embodiment, the level expression of two markers selected in the group of β5 integrin and FAK, or β5 integrin and GSK-3β or FAK and GSK-3β is determining.

In another preferred embodiment, the level expression of the three markers, β5 integrin, FAK and GSK-3β is determining.

In another preferred embodiment, the potential correlation between the level expression of the three markers, β5 integrin, FAK and GSK-3β is determining.

In a preferred embodiment, the level expression of the markers according to the invention is compared with reference level wherein a difference between said levels is indicative of the responsiveness to chemotherapy of said patient.

In a particular embodiment, the reference level may be obtained from responder and non-responder group of patients.

In a preferred embodiment, the chemotherapy is a neoadjuvant chemotherapy. In a particular embodiment, the neoadjuvant chemotherapy is an association of drugs derived from SFOP OS94 regimen see for example Le Deley MC et al, 2007.

Typically, the tumor sample obtained from the patient is a resected ostesarcoma or a biopsy from osteosarcoma.

Typically the level expression of markers may be measured for example by RT-PCR or immunohistochemistry performed on said sample.

In a preferred embodiment, the determination of the level expression of the markers β5 integrin, FAK and GSK-3β in said sample may be performed by determining the gene expression level of the markers β5 integrin, FAK and GSK-3β.

Typically, the determination comprises contacting the sample with selective reagents such as probes, primers or ligands, and thereby detecting the presence, or measuring the amount, of polypeptides or nucleic acids of interest originally in the sample. Contacting may be performed in any suitable device, such as a plate, microtiter dish, test tube, well, glass, column... In specific embodiments, the contacting is performed on a substrate coated with the reagent, such as a nucleic acid array or a specific ligand array. The substrate may be a solid or semi-solid substrate such as any suitable support comprising glass, plastic, nylon, paper, metal, polymers and the like. The substrate may be of various forms and sizes, such as a slide, a membrane, a bead, a column, a gel, etc. The contacting may be made under any condition suitable for a detectable complex, such as a nucleic acid hybrid or an antibody-antigen complex, to be formed between the reagent and the nucleic acids or polypeptides of the sample.

In a particular embodiment, the expression level may be determined by determining the quantity of mRNA. Such method may be suitable to determine the expression level of β5 integrin, FAK or GSK-3β gene in the sample.

Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA may be then detected by hybridization (e. g., Northern blot analysis).

Alternatively, the extracted mRNA may be subjected to coupled reverse transcription and amplification, such as reverse transcription and amplification by polymerase chain reaction (RT-PCR), using specific oligonucleotide primers that enable amplification of a region in the β5 integrin, FAK or GSK-3β gene. Preferably quantitative or semi-quantitative RT-PCR is preferred. Real-time quantitative or semi-quantitative RT-PCR is particularly advantageous. Extracted mRNA may be reverse-transcribed and amplified, after which amplified sequences may be detected by hybridization with a suitable probe or by direct sequencing, or any other appropriate method known in the art.

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization. A wide variety of appropriate indicators are known in the art including, fluorescent, radioactive, enzymatic or other ligands (e. g. avidin/biotin).

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are "specific" to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature Tm, e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

In a particular embodiment, the method of the invention comprise the steps of providing total RNAs obtained from the sample of the patient, and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

Total RNAs can be easily extracted from the sample. For instance, the sample may be treated prior to its use, e.g. in order to render nucleic acids available. Techniques of cell or protein lysis, concentration or dilution of nucleic acids, are known by the skilled person.

In another embodiment, the expression level may be determined by DNA microarray analysis. Such DNA microarray or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

In another preferred embodiment, the determination of the level expression of the markers β5 integrin, FAK and GSK-3β in said sample may be performed by determining the level of β5 integrin, FAK and GSK-3β proteins by immunohistochemistry.

Such methods comprise contacting a sample with a binding partner capable of selectively interacting with β5 integrin, FAK and GSK-3β present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

One preferred method utilizes immunohistochemistry, a staining method based on immunoenzymatic reactions using monoclonal or polyclonal antibodies to detect cells or specific proteins such as tissue antigens. Typically, immunohistochemistry protocols involve at least some of the following steps:

1) antigen retrieval (eg., by pressure cooking, protease treatment, microwaving, heating in appropriate buffers, etc.);

2) application of primary antibody (i.e. anti-β5 integrin oar anti-FAK or anti-GSK-3β antibody) and washing;

3) application of a labeled secondary antibody that binds to primary antibody (often a second antibody conjugate that enables the detection in step 5) and wash;

4) an amplification step may be included;

5) application of a detection reagent (e.g. chromagen, fluorescently tagged molecule or any molecule having an appropriate dynamic range to achieve the level of or sensitivity required for the assay);

6) counterstaining may be used and

7) detection using a detection system that makes the presence of the proteins visible (to either the human eye or an automated analysis system), for qualitative or quantitative analyses.

Various immunoenzymatic staining methods are known in the art for detecting a protein of interest. For example, immunoenzymatic interactions can be visualized using different enzymes such as peroxidase, alkaline phosphatase, or different chromogens such as DAB, AEC, or Fast Red; or fluorescent labels such as FITC, Cy3, Cy5, Cy7, Alexafluors, etc. Counterstains may include H&E, DAPI, Hoechst, so long as such stains are compatable with other detection reagents and the visualization strategy used. As known in the art, amplification reagents may be used to intensify staining signal. For example, tyramide reagents may be used. The staining methods of the present invention may be accomplished using any suitable method or system as would be apparent to one of skill in the art, including automated, semi-automated or manual systems.

The method of the invention may comprise a further step consisting of comparing level of β5 integrin, FAK and GSK-3β with a reference level.

The "reference level" may be the level of the markers determined in a healthy patient, i.e. a patient who does not suffer from any ostesarcoma. The reference level may also be a patient suffering from ostesarcoma. Preferably, said reference level is a healthy patient.

In a particular embodiment, when detection of β5 integrin, FAK or GSK-3β is performed by immunochemistry, the method may further comprise a step consisting of determining the amount of cells that express β5 integrin, FAK or GSK-3β (" β5 integrin+ cells", "FAK+ cells," or "GSK-3β+ cells").

In a preferred embodiment a percentage of β5 integrin+ cells, FAK+ cells, or GSK-3β+ cells of at least 20%, preferably at least 21%, more preferably at least 22%, even more preferably at least 23%, at least 24%, at least 25%, at least 26%, at least 27%, preferably at

least 28%, more preferably at least 29%, even more preferably 30% and more preferably 50% is indicative of a poor responder.

In other words, patients with low percentage of β5 integrin+ cells, FAK+ cells, or GSK-3β+ is indicative of a good response of a patient affected with an osteosarcoma to chemotherapy.

In a particular embodiment, the step of determining the amount of β5 integrin+ cells, FAK+ cells, or GSK-3β+ cells may be combined with a step of determining the staining intensity of the β5 integrin+ cells, FAK+ cells, or GSK-3β+ cells.

Because the inventors showed a protein profile of prediction of sensitivity to chemotherapy, the present invention also relates to kits for performing the method according to the invention.

The present invention also relates to kits for performing the method according to the invention comprising means for determining the level of beta5 integrin, FAK and GSK-3 beta expression in tumor sample.

According to the invention, kits of the invention may comprise 3 antibodies directed against beta5 integrin, FAK and GSK-3 beta and another molecule coupled with a signalling system which binds to said antibodies.

Typically, the antibodies or combination of antibodies are in the form of solutions ready for use. In one embodiment, kits comprise containers with the solutions ready for use. Any other forms are encompassed by the present invention and the man skilled in the art can routinely adapt the form to the use in immunohistochemistry.

Enhancement of clinical efficiency of chemotherapy

A second object of the invention relates to a compound which is an antagonist of β5 integrin, FAK or GSK-3β or an inhibitor of the expression of β5 integrin, FAK or GSK-3β for use in a method for enhancing clinical efficiency of chemotherapy.

In a preferred embodiment, said compound according to the invention is a β5 integrin, FAK or GSK-3β antagonist.

In one embodiment, the compound according to the invention may bind to β5 integrin, FAK or GSK-3β and block their physiological effects. To identify a compound able to block

these molecules, a survival as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test performed on osteosarcoma cell lines, which demonstrates the agonist or synergistic effect between at least one of these inhibitors and any chemotherapy drug used in the derived SFOP 0S94 egimen protocol may be used.

In one embodiment, said β5 integrin, FAK or GSK-3β antagonist may be a low molecular weight antagonist, e. g. a small organic molecule (natural or not).

The term "small organic molecule" refers to a molecule (natural or not) of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e. g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 10000 Da, more preferably up to 5000 Da, more preferably up to 2000 Da and most preferably up to about 1000 Da.

Antagonists of β5 integrin are well known in the state of the art (see for example Desgrosellier JS, et al, 2010).

In a particular embodiment, the antagonist of β5 integrin according to the invention is SCH221153 (see for example Kumar CC et al, 2001).

In another particular embodiment, the antagonist of β5 integrin according to the invention is SB-267268 (see for example Wilkinson-Berka J et al, 2006).

In another embodiment, the antagonist of β5 integrin according to the invention is the Cilengitide (see for example Reardon et al, 2008).

Antagonists of FAK are well known in the state of the art (see for example Schultze A. et al. 2010, Expert Opin. Investig. Drugs).

In a particular embodiment, the antagonist of FAK according to the invention is TAE226 (see for example Jyotsnabaran H et al., 2007) or the 1,2,4,5-Benzenetetraamine tetrahydrochloride (see for example Golubovskaya V et al., 2008).

Antagonists of GSK-3β are well known in the state of the art (see for example Kypta R, 2005, Expert Opin. Ther. Patents or Dorronsoro I et al, 2002, Expert Opin. Ther. Patents).

In a particular embodiment, the antagonist of GSK-3β according to the invention is a thiadiazolidinone (see for example Zou Y et al., 2008).

In a particular embodiment, the antagonist of GSK-3β according to the invention is pyrimidine and pyridine derivatives or substitutes (see for example patent applications US2009074886, WO0220495 or WO 2010012398).

In a particular embodiment, the antagonist of GSK-3β according to the invention is the 3-benzofuranyl-4-indolyl maleimides (see for example patent application WO2008077138).

In another embodiment, β5 integrin, FAK or GSK-3β antagonist of the invention may be an anti-β5 integrin antibody or an anti-FAK antibody or an anti-GSK-3β antibody which neutralizes β5 integrin, FAK or GSK-3β or an anti-β5 integrin fragment thereof or an anti-FAK fragment or anti- GSK-3β fragment which neutralizes β5 integrin, FAK or GSK-3β.

Antibodies directed against β5 integrin, FAK or GSK-3β can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies against β5 integrin, FAK or GSK-3β can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975); the human B-cell hybridoma technique (Cote et al., 1983); and the EBV-hybridoma technique (Cole et al. 1985). Alternatively, techniques described for the production of single chain antibodies (see e.g., U.S. Pat. No. 4,946,778) can be adapted to produce anti-β5 integrin, anti-FAK or anti-GSK-3β single chain antibodies. β5 integrin, FAK or GSK-3β antagonists useful in practicing the present invention also include antibody fragments including but not limited to F(ab')2 fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to β5 integrin, FAK or GSK-3β.

Humanized anti-β5 integrin, anti-FAK or anti-GSK-3β antibodies and antibody fragments therefrom can also be prepared according to known techniques. "Humanized antibodies" are forms of non-human (e.g., rodent) chimeric antibodies that contain minimal

sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (CDRs) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Methods for making humanized antibodies are described, for example, by Winter (U.S. Pat. No. 5,225,539) and Boss (Celltech, U.S. Pat. No. 4,816,397).

Then, for this invention, neutralizing antibodies of β5 integrin, FAK or GSK-3β are selected.

In still another embodiment, β5 integrin, FAK or GSK-3β antagonists may be selected from aptamers. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

Then, for this invention, neutralizing aptamers of β5 integrin, FAK or GSK-3β are selected.

In a preferred embodiment, the compound according to the invention is an inhibitor of the β5 integrin, FAK or GSK-3β gene expression.

Small inhibitory RNAs (siRNAs) can also function as inhibitors of β5 integrin, FAK or GSK-3β gene expression for use in the present invention. β5 integrin, FAK or GSK-3β gene expression can be reduced by contacting a 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 β5 integrin, FAK or GSK-3β gene 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 for example Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); 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. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of β5 integrin, FAK or GSK-3β gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of 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 β5 integrin, FAK or GSK-3β mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are 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 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 inhibitors of β5 integrin, FAK or GSK-3β gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite 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 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 β5 integrin, FAK or GSK-3β. 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. 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 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. 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 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, 1990 and in Murry, 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 hemopoietic 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. Sambrook et al., 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, pUCl9, 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, eye, 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 a preferred embodiment, the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a

heterologous promoter. The promoter may be specific for Muller glial cells, microglia cells, endothelial cells, pericyte cells and astrocytes For example, a specific expression in Muller glial cells may be obtained through the promoter of the glutamine synthetase gene is suitable. The promoter can also be, e.g., a viral promoter, such as CMV promoter or any synthetic promoters.

Another object of the invention relates to a method for treating ostesarcoma comprising administering to a subject in need thereof a therapeutically effective amount of compound which is an antagonist of β5 integrin, FAK or GSK-3β or an inhibitor of the expression of β5 integrin, FAK or GSK-3β as described above.

In one aspect, the invention relates to a method for treating osteosarcoma comprising administering to a subject in need thereof a therapeutically effective amount of a β5 integrin, FAK or GSK-3β antagonist as above described.

Compounds of the invention may be administered in the form of a pharmaceutical composition, as defined below.

By a "therapeutically effective amount" is meant a sufficient amount of compound to treat and/or to prevent ostesarcoma disorder.

It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment and/or during phase 1 clinical try. 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 compound 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 compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide 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 compound 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. Preferably, 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 active ingredient 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 active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. 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.

Compounds according to the invention may be used for the preparation of a pharmaceutical composition for the treatment of ostesarcoma.

Hence, the present invention also provides a pharmaceutical composition comprising an effective dose of an antagonist of β5 integrin, FAK or GSK-3β or an inhibitor of the β5 integrin, FAK or GSK-3β expression, preferably a β5 integrin, FAK or GSK-3β antagonist, according to the invention.

Any therapeutic agent of the 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" refers 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.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, parenteral, intraocular, intravenous, intramuscular or subcutaneous administration and the like.

Preferably, 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 doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

In addition, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently can be used.

Compositions of the present invention may comprise a further therapeutic active agent. The present invention also relates to a kit comprising an antagonist or an inhibitor according to the invention and a further therapeutic active agent.

In one embodiment said therapeutic active agent is an anticancer agent. For example, said anticancer agents include but are not limited to fludarabine, gemcitabine, capecitabine, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbizine, etoposide, teniposide, campathecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, doxorubicin, epimbicm, 5-fluorouracil, taxanes such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustme and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatimb mesylate, hexamethyhnelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrphostins, protease inhibitors, inhibitors herbimycm A, genistein, erbstatin, and lavendustin A. In one embodiment, additional anticancer agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates, pyrimidine analogs, purine analogs, DNA antimetabolites, taxanes, podophyllotoxin, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimitotic agents, isoprenylation inhibitors, cell cycle inhibitors, actinomycins, bleomycins, anthracyclines, MDR inhibitors and Ca2+ ATPase inhibitors.

Additional anticancer agents may be selected from, but are not limited to, cytokines, chemokines, growth factors, growth inhibitory factors, hormones, soluble receptors, decoy receptors, monoclonal or polyclonal antibodies, mono-specific, bi-specific or multi-specific antibodies, monobodies, polybodies.

Additional anticancer agent may be selected from, but are not limited to, growth or hematopoietic factors such as erythropoietin and thrombopoietin, and growth factor mimetics thereof.

In the present methods for treating cancer the further therapeutic active agent can be an antiemetic agent. Suitable antiemetic agents include, but are not limited to,

metoclopromide, domperidone, prochlorperazine, promethazine, chlorpromazine, trimethobenzamide, ondansetron, granisetron, hydroxyzine, acethylleucine monoemanolamine, alizapride, azasetron, benzquinamide, bietanautine, bromopride, buclizine, clebopride, cyclizine, dunenhydrinate, diphenidol, dolasetron, meclizme, methallatal, metopimazine, nabilone, oxypemdyl, pipamazine, scopolamine, sulpiride, tetrahydrocannabinols, thiefhylperazine, thioproperazine and tropisetron. In a preferred embodiment, the antiemetic agent is granisetron or ondansetron.

In another embodiment, the further therapeutic active agent can be an hematopoietic colony stimulating factor. Suitable hematopoietic colony stimulating factors include, but are not limited to, filgrastim, sargramostim, molgramostim and epoietin alpha.

In still another embodiment, the other therapeutic active agent can be an opioid or non-opioid analgesic agent. Suitable opioid analgesic agents include, but are not limited to, morphine, heroin, hydromorphone, hydrocodone, oxymorphone, oxycodone, metopon, apomorphine, nomioiphine, etoipbine, buprenorphine, mepeddine, lopermide, anileddine, ethoheptazine, piminidine, betaprodine, diphenoxylate, fentanil, sufentanil, alfentanil, remifentanil, levorphanol, dextromethorphan, phenazodne, pemazocine, cyclazocine, methadone, isomethadone and propoxyphene. Suitable non-opioid analgesic agents include, but are not limited to, aspirin, celecoxib, rofecoxib, diclofinac, diflusinal, etodolac, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, indomethacin, ketorolac, meclofenamate, mefanamic acid, nabumetone, naproxen, piroxicam and sulindac.

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: Illustration of immunohistochemical staining for several biological markers in controls and osteosarcomas (OS).

Sections stained were photographed at × 400 magnification (B, C, D, E, F, H and J) or x 200 magnification (A, G and I).

A. Colon lining cells serves as a positive control for integrin β5 staining.

B. Integrin β5 immunostaining in OS.

C. Breast adenocarcinoma serves as a positive control for GSK3- β staining.

D. GSK3- β immunostaining in OS.

E. Breast adenocarcinoma serves as a positive control for FAK staining

F. FAK immunostaining in OS.

G. Lung adenocarcinoma serves as a positive control for RhoB staining.

H. RhoB immunostaining in OS.

I. Parietal cells of the stomach serve as a positive control for ezrin staining.

J. Ezrin immunostaining in OS.

Figure 2: Kaplan Meier estimation.

A. Metastasis free survival according to the expression of Exzin.

B. Overall survival according to the expression of β5 integrin.

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Clinical characteristics	Values N	%
Age at diagnostic (years)		
Median	18.0	
Range	8.0:77.0	
Gender(number of patients)		
Female	18	(43.9)
Male	23	(56.1)
High grade osteosarcoma (subtype)		
osteoblastic	8	(19.5%)
chondroblastic	13	(31.7%)
fibroblastic	12	(29.3%)
Mixed	6	(9.7%)
Other	2	(4.9%)
Tumor Size		
<= 5cm	6	(15.0%)
> 5 cm	34	(85.0%)
Missing	1	
Tumor location(number of patients)		
Long bone	31	(77.5)
Flat bone	13	(15.0)
other	3	(7.5)
Missing	1	
***Histologic response** (number of patients)		
Good responders	20	(54.1)
Poor responders	17	(45.9)
Metastatic disease at diagnosis (number of patients)		
Absent	40	(97.6)
Present	1	(2.4)
Surgical treatment (number of patients)		
Wide conservative surgery	36	
Amputation	1	
Limb salvage	36	
Microscopically complete resection (R0)	36	

Table 1: Clinical characteristics and treatment modalities (Data between brackets are percentages).*Evaluation performed on the 37 patients who underwent surgery after neo-adjuvant chemotherapy.

	Poor responder N %	Good responder N %	p	AUC IC95%
Beta Catenin (IRS)			p = 0.6297	54.8%
Median	4.0	4.0		[35.2;74.4]
(Range)	(0.0: 12.0)	(0.0: 12.0)		
Missing	0	3		
GSK3 (IRS)			*p = 0.0861*	
Median	6.0	2.5		66.9%
(Range)	(2.0: 12.0)	(0.0: 9.0)		[49.1;84.9]
Missing	0	2		
ILK (IRS)			p = 0.2879	
Median	6.0	3.0		60.8%
(Range)	(2.0: 12.0)	(0.0: 12.0)		[40.9;80.7]
Missing	1	3		
FAK (IRS)			p = 0.0166	
Median	6.0	3.0		74.4%
(Range)	(0.0: 9.0)	(0.0: 8.0)		[56.9;91.9]
Missing	1	3		
Beta1 (IRS)			p = 0.9450	
Median	6.0	6.0		50.7%
(Range)	(2.0: 12.0)	(3.0: 12.0)		[30.8;70.6]
Missing	1	2		
Beta3 (IRS)			p = 0.9176	
Median	4.0	3.5		51.0%
(Range)	(0.0: 12.0)	(0.0: 12.0)		[31.3;70.7]
Missing	1	2		
Beta5 (IRS)			p = 0.1047	
Median	12.0	6.0		66.1%
(Range)	(0.0: 12.0)	(0.0: 12.0)		[47.3;84.9]
Missing	1	1		
RhoB (IRS)			p = 0.8494	
Median	2.0	2.5		51.9%
(Range)	(0.0: 12.0)	(0.0: 6.0)		[31.0;72.9]
Missing	3	2		
MMP9 (IRS)			p = 0.6911	
Median	10.5	12.0		53.9%
(Range)	(6.0: 12.0)	(3.0: 12.0)		[34.5;73.4]
Missing	1	1		

Ang2 (IRS)		p = 0.9587	50.5%
Median	5.0	3.0	[30.6;70.4]
(Range)	(2.0: 6.0)	(2.0: 8.0)	
Missing	1	2	
Ezrin (IRS)		p = 0.7015	53.8%
Median	3.0	4.5	[34.2;73.4]
(Range)	(0.0: 12.0)	(0.0: 12.0)	
Missing	2	0	

Table 2: Univariate Analysis : Relationship between biological markers and response to chemotherapy.

	Overall Survival (n=41)			Metastasis Free Survival (n=40)		
	Event/N	S(5y)*	p	Event/ N	S(5y)	p
Age at diagnosis	:	*p=0.0876*				p = 0.1302
<18 years	4 / 18	0.833		4 / 17	0.749	
>=18 years	10 / 23	0.425		10 / 23	0.519	
Gender			p=0.9429			p = 0.3624
Female	7 / 18	0.658		8 / 18	0.543	
Male	7 / 23	0.567		6 / 22	0.695	
Histologic Tumor Size			p=0.8785	:		p = 0.4053
<=5 cm	2 / 6	0.556		1 / 6	0.833	
>5cmm	12 / 34	0.619		13 / 33	0.576	
Response to chemotherapy			p=0.0145			*p = 0.0855*
Poor	9 / 17	0.431		8 / 16	0.391	
Good .	4 / 20	0.788		5 / 20	0.750	

* Kaplan-Meier estimation at 5 years.

Table 3: Univariate analysis for overall survival and metastasis Free Survival

	HR	IC95%	p
Metastasis Free Survival			
Response to Chemotherapy			
Poor responder	3.36	[0.99;11.39]	0.052
Good responder	1		
Ezrin IRS			
Low Expression	1		
High Expression	4.21	[1.27;13.93]	0.019
Overall Survival			
Response to Chemotherapy			
Poor responder	2.63	[0.74;9.30]	0.132
Good responder	1		
Age at diagnosis			
<18 years	1		

>=18 years	1.85	[0.53;6.43]	0.332
Beta 5 integrin			
Low Expression	1		
High Expression	1.84	[0.52;6.61]	0.345

Table 4: Multivariate analysis for overall survival and metastasis free survival using cox proportional hazard modelling

EXAMPLE:

Material & Methods

1.1 Patients and tumour characteristics

We studied in the department of pathology of the University Hospital of Toulouse, France, forty one osteosarcomas from patients treated by SFOP OS94 derived chemotherapy regimen at the Comprehensive Cancer Center Claudius Regaud of Toulouse and the oncologic pediatric department of the Purpan hospital, France, and who underwent post-chemotherapy surgical resection at the orthopedic surgery departments of the hospitals of Toulouse (Purpan and Rangueil), between April 1996 and April 2006. All the 41 osteosarcomas were of high grade, one patient had metastatic disease at inclusion. The diagnosis was performed on the initial biopsy and analysed by the experts of the French group of Bone Pathologists (GFPO). Histological diagnosis was conventional osteosarcoma type in all cases. All patients provided written informed consent to use the biopsy samples for research purposes. Complete clinical information and tissue blocks from the biopsy specimens, before any treatment, were available for the 41 patients. Thirty seven patients received preoperative and postoperative chemotherapy derived from SFOP OS94 regimen [Le Deley et al, 2007]. Four patients didn't have surgical resection because of the presence of metastases at diagnosis for one case and because of the poor clinical status of the three other patients. The response to preoperative chemotherapy was assessed on the surgical resection, according to Rosen's protocol on 37 patients [Rosen et al, 1982]. No more than 10% viable tumours defined good responders. All data, including age, gender, histologic features, tumour stage and follow-up were obtained from the clinical and pathologic records.

1.2 Construction of tissue microarrays

Tissue microarrays (TMAs) were made by the pathology center (IFR30, U563, CHU Purpan, Toulouse) from 41 selected surgical preoperative chemotherapy biopsies, according to the TMA technique developed by Kononen [Kononen et al, 1998]. Briefly, tissues samples were fixed in 10% buffered formalin, decalcified and embedded in paraffin wax. Representative areas of each tumour were selected for TMA production by first observing the biopsy tumour slide stained with hematoxylin and eosin (H&E) and then collecting the tissue from corresponding paraffin blocks. A tissue microarray instrument (Beecher Instruments, Sun Prairie, WI, USA) was used to create holes in the different paraffin blocks and, for each tumour, three tissue cores of 1-2 mm diameter, representative of the tumour samples, were taken from the primary paraffin block and placed in a new paraffin block. Serial sections of 4µm thickness were cut from the TMA blocksby using a conventional microtome. One section was stained immediately with H&E, whereas the remaining sections were stored at room temperature before immunostaining. All samples were used in compliance with the French bioethics laws regarding patient information and consent.

1.3 Immunohistochemistry

Sets of 4µm thick section from the TMA blocks were transferred to adhesive-coated slides. The TMA sections containing samples of the 41 bone tumours were deparaffinised and rehydrated. Heat antigen retrieval was used with citrate buffer (PH 6) for β3 integrin, Ang-2 and Ezrin. For the others antibody, sections were incubated with EDTA buffer (1mM, PH 8 or PH 9) for 40 min at 98°C to renature the antigen. Then sections were incubated for 5 min in 3% hydrogen peroxide to quench endogenous peroxidase activity. Next sections were incubated with the following antibodies for 60 min at room temperature: β1 integrin (Imgenex , polyclonal, dilution 1:200), β3 integrin (Imgenex , polyclonal, dilution 1:200), β5 integrin (Abcam, polyclonal, dilution 1:50), ILK (Santa Cruz Biotechnology (65.1) monoclonal, dilution 1:200), FAK (Biosource (44-624G) polyclonal, dilution 1: 50), MMP-9 (R/D system (4H3) monoclonal, dilution 1:50), RhoB (Santa Cruz Biotechnology (C-5), monoclonal, dilution 1:20) , Ang-2 (Santa Cruz Biotechnology, polyclonal, dilution 1:50), β-catenin (Dako (M3539) monoclonal, dilution 1:200), GSK-3β (Cell Signaling (3C12) polyclonal, dilution 1:100), Ezrin (Sigma,(E 8897) monoclonal, dilution 1:50). These sections were incubated with biotinylated link antibody and then with peroxydase-labeled streptavidin (LSAB™ + Kit; Dako, Trappes, France). Staining was completed by incubation with diaminobenzidine solution (DAB liquid; Dako). Omitting the specific primary antibody performed negative controls. Positive controls were chosen according to the studied protein:

megakaryocyte for β3 integrin staining, basal cell of epiderm for β1 integrin staining, colonic crypts for β5 integrin staining, stomach parietal cells for Ezrin staining, bone metastasis of adenocarcinoma (lung or breast) for FAK, MMP-9 RhoB, GSK-3β staining, desmoid tumor for β-catenin staining and vascular endothelium for Ang-2 and ILK staining.

An immunoreactive score (IRS) was assessed, combining the percentage of positive tumour cells and staining intensity [Remmele et al, 1987]. Staining intensity was evaluated as 0=negative, 1=weak, 2=moderate and 3=strong. The percentage of labelled cells was categorized using a five-point scale i.e. 0=0%, 1=1 to 10%, 2=11 to 50%, 3=51 to 80%, 4=81 to 100%. The IRS (from 0 to 12) was obtained by multiplying intensity and percentage scores. All the immunostains were independently evaluated by 2 pathologists. Concordance between the two pathologists was estimated using the intraclass correlation coefficient. As this study show a high reproducibility, statistical analysis was performed with the results obtain by the senior pathologist (data not shown).

1.4 Statistical analysis

Correlations between continuous variables were evaluated using Spearman's rank correlation test. The comparison of the different markers between "good" and "poor" responders was done with the use of a nonparametric Man-Whitney test. Differences were considered statistically significant when $P < 0.05$. Multivariate analysis is based on receiver operating characteristic curves, which allow the characterization of the discrimination between two well-defined populations. The generalized receiver operating characteristic criterion [Kramar et al, 2001 and Reiser et al, 1997] finds the best linear combination (virtual marker) of the tumour markers such that the area under the curve is maximized. Sensitivity, which represents its ability to detect the diseased population, and specificity, which represents its ability to detect the responder population, for individual and combined marker performance, was evaluated with the use of the optimal threshold value calculated to maximize the Youden's index. This index is defined as the sum of sensitivity and specificity minus one. Over-expression was defined when IRS value was higher than the optimal cut-off.

The cut-off of the markers used in survival analysis correspond to those defined using youden index on response. All survival times were calculated from the date of diagnosis using the following first-event definitions: distant recurrence for metastasis-free survival (MFS) and death from any cause for overall survival (OS). MFS and OS were estimated by the Kaplan-Meier method and univariate analysis were performed using Logrank test to identify prognostic factors associated. All factors considered significant at the $P<0.05$ level by this

method were included in a Cox multivariate analysis to identify the major independent prognostic factors.

All P-values reported were two-sided. For all statistical tests, differences were considered significant at the 5% level. Statistical analyses were performed using STATA 11.0 software.

Results

3.1 Patients characteristics

Table 1 lists the demographic, clinical and clinical outcomes of the 41 patients included in this study. The 41 osteosarcomas were conventional high grade osteosarcoma according to the WHO classification of 2002. Among them, 8 were osteosblastic osteosarcomas, 13 were chondroblastic osteosarcomas, 12 were fibroblastic osteosarcoma and 4 could not be assigned to a specific subgroup due to unusual morphological features (such as predominant giant cells, predominant storiform pattern). Thirty-seven patients were treated by neo-adjuvant chemotherapy before surgery. Among them, seventeen (45.1 %) had less than 90 % tumour necrosis following induction therapy and were classified as poor responders (Huvos grade I/II), 20 had more than 90 % or complete necrosis and were classified as good responders (Huvos grade III/IV). Thirty-six patients had wide conservative surgery with microscopically complete resection (R0), and one had an amputation. After median follow-up of 70 months (95 % CI [48.7; 98.3]), eighteen patients presented metastases during the course of the disease with 17 located in lungs and one located in bone. The five years metastases free survival was estimated to 62.1 % (95 % CI =[43.8;76.0]. At the last follow-up fourteen patients were dead of their disease. The five year overall survival was 63.67% (95 % CI =[45.0;77.4]).

3.2 Relationship between biologic and clinicopathologic parameters

We performed an immunohistochemical analysis for the detection of β-catenin (n = 37), GSK-3β (n = 38), ILK (n = 37), FAK (n = 36), β1 integrin (n = 37), β3 integrin (n = 37), β5 integrin (n = 38), RhoB (n = 35), MMP9 (n = 38), Ang-2 (n = 37) and Ezrin (n = 37). Staining was detected in the membrane and cytoplasm of tumour cells for β1 integrin, β3 integrin, β5 integrin and β-catenin. For GSK-3β, ILK, FAK, RhoB, MMP9, Ang-2 and Ezrin, the staining was detected in tumour cell cytoplasm. Among osteosarcoma biopsies analyzed, 12 tumours (32.4%) overexpressed β-catenin, 16 tumors (43.2%) overexpressed β1 integrin,

19 tumours (51.4%) overexpressed β3 integrin, 17 tumours (44.7%) overexpressed β5 integrin (figure 1B), 32 tumours (84.2%) overexpressed GSK-3β (figure 1D) , 23 tumours (63.9%) overexpressed ILK, 15 tumours (4.17%) overexpressed FAK (Figure 1F), 17 tumours (48.6%) overexpressed RhoB (figure 1H), 21 tumours (55.3%) overexpressed MMP9, 18 tumours (48.6%) overexpressed Ang-2, 12 tumours (32.4%) overexpressed Ezrin (figure 1J).

We first investigated whether a relationship might exist between the biologic and clinicopathologic parameters. No correlation was shown between the studied markers expressions and the clinicopathological parameters (age, gender) (data not shown).

We then studied the potential links between the biologic parameters. FAK expression was statistically linked with ILK expression (rho = 0.43; p = .0109) and β1 integrin expression (rho = 0.49); p = .0026) was also significantly linked with FAK expression. Moreover, a significant association between RhoB and ILK expression was found (rho = 0.48; p = .0042), suggesting that a regulation of β1 integrin, RhoB and ILK expression might exist in high grade osteosarcoma. Finally a strong statistical link exists in our cohort between β1 integrin expression and β3 integrin expression (rho = 0,60; p = .0002).

3.4 Relationship between biologic parameters and therapeutic outcomes

We next evaluated the correlation of the selected markers with the different therapeutic outcomes: response to preoperative chemotherapy, metastasis free survival and overall survival.

Response to Chemotherapy

No correlation was found between response and age at the diagnosis (p = 0.266), gender (p = 0.230), histologic subtypes (p = 0.308) and the tumour size (p = 1.0).

We then investigated whether potential relationships may exist between the expression of these proteins on preoperative chemotherapy biopsies and the response to chemotherapy ("good" or "poor" responders assessed on resected specimens).

Univariate analysis

As shown in table 2, good responders were statistically associated with lower FAK expression (median = 3, range = [0;8]) than poor responders (Median = 6, range = [0;9], p = 0.016). The receiver operating characteristic area under the curve (AUC), estimated to determine the discriminate value of each marker was of 0.74 for the marker FAK [95% confidence interval [0.57-0.92]) when we compared poor and good responders. The optimal

cut-off of FAK, determined with the Younden 's indice, was 3. If the IRS score of FAK is higher than 3, the number of poor responders is higher, suggesting that FAK is a pejorative marker. Sensitivity, specificity and diagnostic accuracy of FAK expression were respectively estimated to 82.2%, 68.5% and 75.8%.

Good responders tended to have a lower expression of GSK-3β (median=6; range=[2;12], p=0.086) compared to poor responders (median=2.5,range=[0;9]). The AUC was 0.67 (95% CI = [0.50;0.85). At the optimal cut-off, estimated to be 1, GSK-3β, achieved a sensitivity of 33.3%, specificity of 100.0% and diagnostic accuracy of 65.71%. Using this cut-off, GSK-3β expression was associated to a pejorative response to treatment.

Good responders tended to have a lower expression of β5 integrin (median=6, range = [0;12]; p = 0.10) than poor responders (median=12; range=[0;12]). The AUC was 0.66 (95% CI = 0.47;0.85]). At the optimal threshold, estimated to be 9 for β5 integrin, sensitivity, specificity and diagnostic accuracy were respectively estimated to 73.7%, 62.5% and 68.6%. FAK overexpression was stastistically associated with a poor response, GSK-3β and β5 integrin tend to be pejorative markers for the response to treatment. No significant statistical correlation was found between the response to chemotherapy and the others parameters studied by immuno-histochemistry.

Multivariate analysis

Based on the results obtained on the univariate analysis, we then studied the association of FAK, GSK-3β and β5 integrin with the response to chemotherapy in a multivariate analysis.

Interestingly, the combination of the three markers β5 integrin, FAK and GSK-3β was able to discriminate good and poor responder to chemotherapy with the highest area under the curve (0.90%; 95% CI = [0.71;0.97], yielding a sensitivity of 94.1%, a specificity of 86.7% and a diagnostic accuracy of 90.2%.

These results clearly show that the β5 integrin, FAK and GSK-3β expression profile in high grade ostesarcoma is associated with the poor response to chemotherapy.

Metastasis Free Survival

On the univariate analysis, only response to chemotherapy was statistically associated with metastases free survival (p = 0.01) (Table 3).

Concerning the biological markers, only overexpression of Ezrin was statistically associated with an increased risk of metastasis (p=0.0175) (figure 2 A). Five years metastasis free survival rates were respectively estimated to 73.3% and 40.0% in the group with low and high expression of Ezrin. This result was confirmed in multivariate analysis (Table 4). The hazard ratio of distant metastasis was estimated to 4.21 (95% CI = [1.27;13.93], p=0.019) for patients who overexpressed Ezrin compared to others.

Overall Survival

No statistically association was found between overall survival and clinico-pathological factors (data not shown).

In univariate analysis, concerning overall survival, only over-expression of β5 integrin was statistically associated with an increased risk of death (p=0.0419) (figure 2B). The estimated 5-years overall survival rates were 80.4% and 43.1% for patients with low and high expression of β5 integrin. No significant correlation was found between other proteins and overall survival. In multivariate analysis (Table 4), no correlation was found between β5 integrin expression and overall survival (HR=1.84, 95%CI=[0.52;6.61] p=0.345).

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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. An ex vivo method for predicting the responsiveness of a patient affected with an osteosarcoma to chemotherapy, comprising the step of determining the level expression of at least one marker selected in the group consisting of β5 integrin, FAK or GSK-3β in a tumor sample from said patient.

2. The ex vivo method according to claim 1 wherein the level expression of at least two markers is determined.

3. The ex vivo method according to claims 1 wherein the level expression of three markers is determined.

4. The ex vivo method according to the claims 1 to 3 wherein the level expression of said markers is compared with reference level wherein a difference between said levels is indicative of the responsiveness to chemotherapy of said patient.

5. The ex vivo method according to claims 1 to 4, wherein the tumor sample is selected from the group consisting of a resected osteosarcoma, or a biopsy from osteosarcoma.

6. The ex vivo method according to claim 1 to 5 wherein the level expression of the markers or the combination of marker is made by immunohistochemistry.

7. A compound which is an antagonist of β5 integrin, FAK or GSK-3β or an inhibitor of the expression of β5 integrin, FAK or GSK-3β for use in a method for enhancing clinical efficiency of chemotherapy.

8. A pharmaceutical composition for use in the treatment of osteosarcoma comprising a compound according to claims 7 and a pharmaceutically acceptable carrier.

9. A kit for performing the method according to claim 1 which comprises means for determining the level of beta5 integrin, FAK and GSK-3 beta expression in a tumor sample.

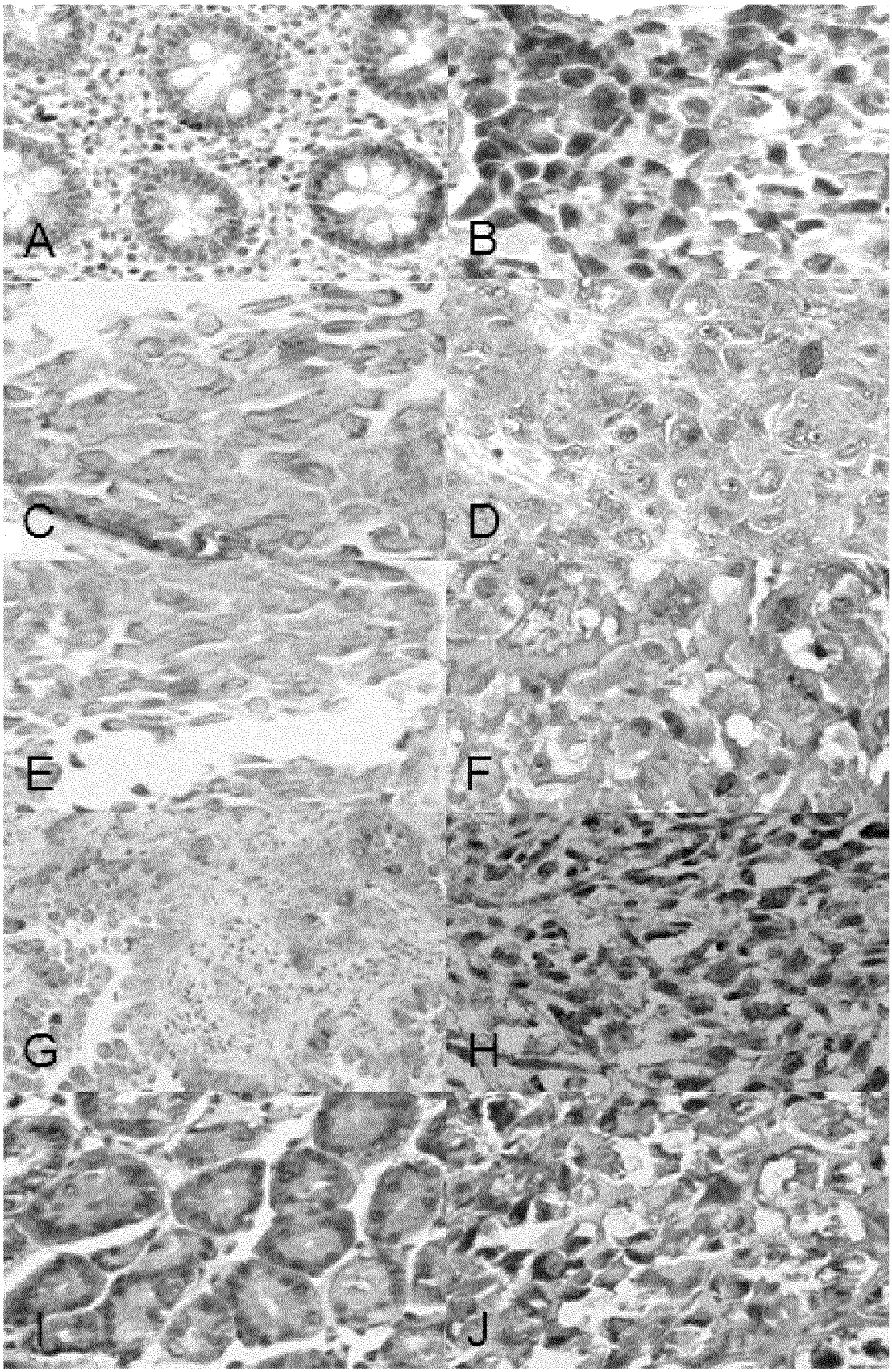
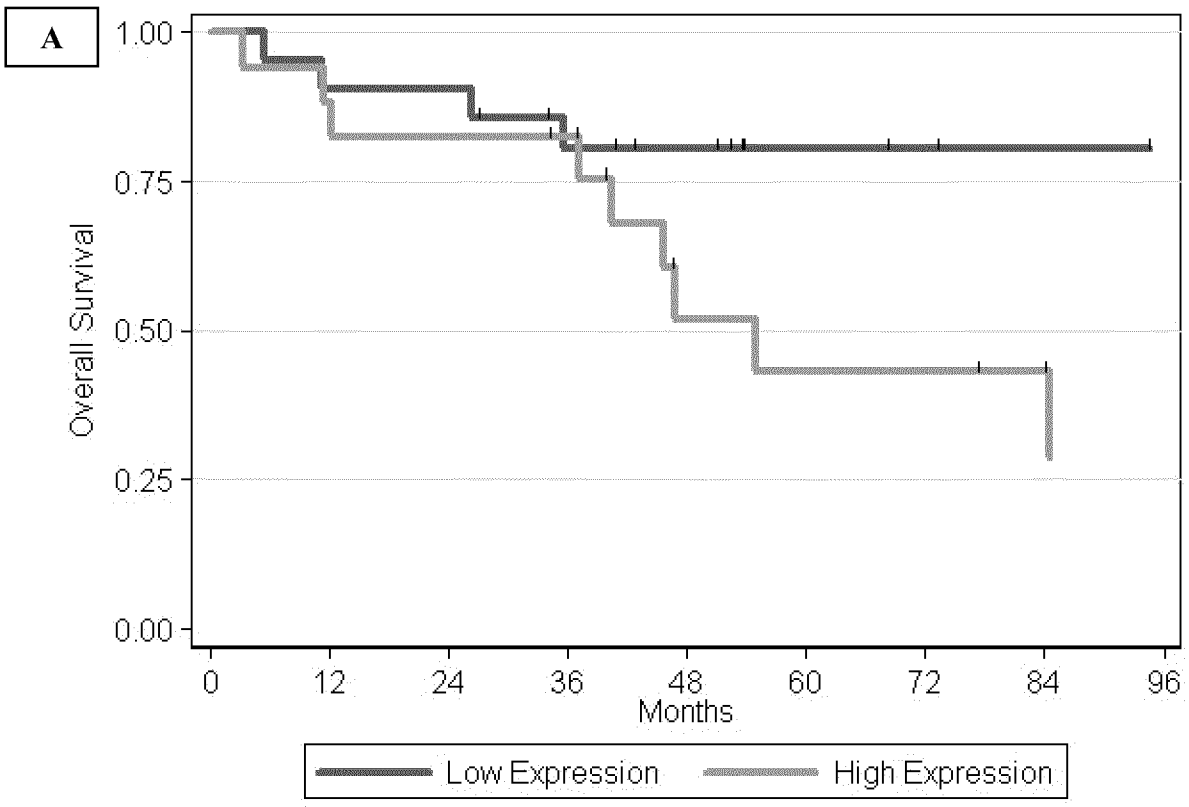


Figure 1



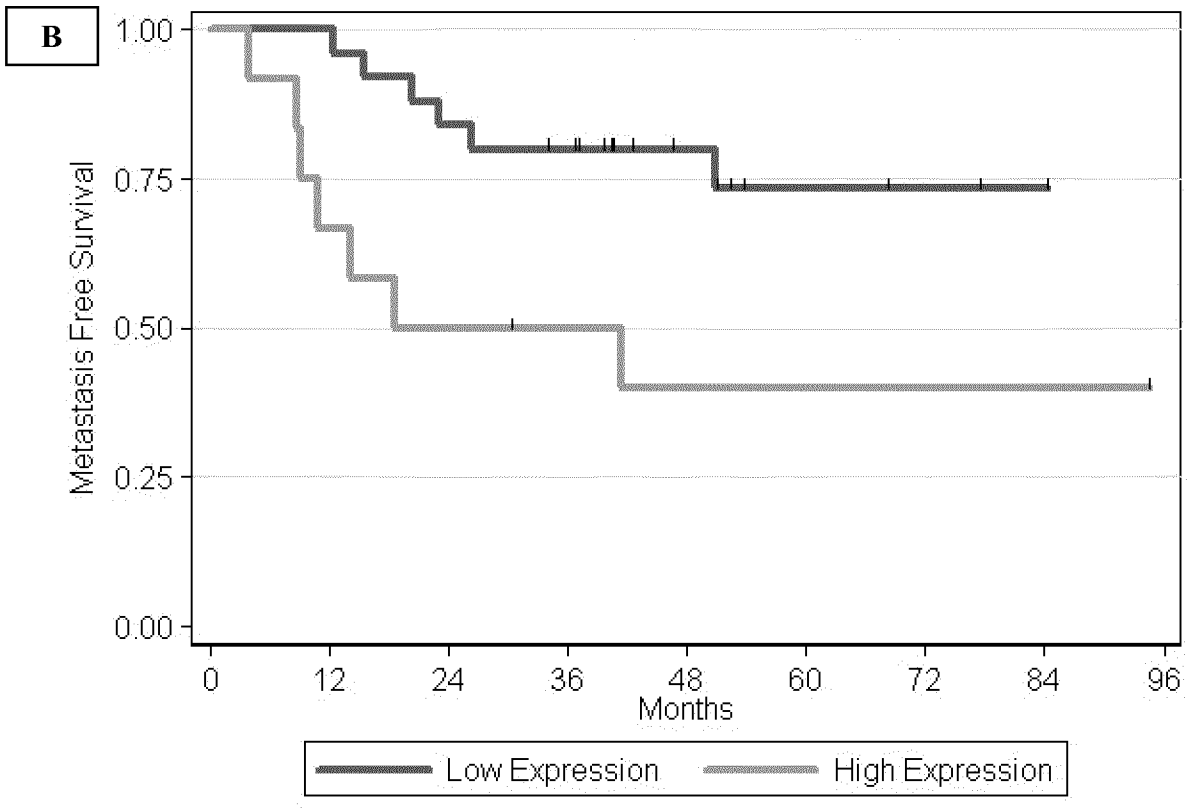


Figure 2 A and B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/062147

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, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2012	07/08/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Moreno de Vega, C

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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