Abstract:
The invention provides newly identified proteins as markers for the detection of tumors, or as targets for their treatment, particularly of tumors affecting lung, colon, breast, ovary; affinity ligands capable of selectively interacting with the newly identified markers; methods of screening a tissue sample for malignancy, for determining the presence of a tumor in a subject and for screening a test compound as an antitumor candidate; a diagnostic kit.
Published:
— with international search report (Art. 21(3))
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2Qi))

— with sequence listing part of description (Rule 5.2(a))
TUMOR MARKERS AND METHODS OF USE THEREOF

The present invention relates to newly identified proteins as markers for the detection of tumors, or as targets for their treatment, particularly of tumors affecting lung, colon, breast and prostate. Also provided are affinity ligands capable of selectively interacting with the newly identified markers, as well as methods for tumor diagnosis and therapy using such ligands.

Background of the invention

Tumor markers (or biomarkers)

Tumor markers are substances that can be produced by tumor cells or by other cells of the body in response to cancer. In particular, a protein biomarker is either a single protein or a panel of different proteins that could be used to unambiguously distinguish a disease state. Ideally, a biomarker would have both a high specificity and sensitivity, being represented in a significant percentage of the cases of given disease and not in healthy state.

Biomarkers can be identified in different biological samples, like tissue biopsies or preferably biological fluids (saliva, urine, blood-derivatives and other body fluids), whose collection does not necessitate invasive treatments. Tumor marker levels may be categorized in three major classes on the basis of their clinical use. Diagnostic markers can be used in the detection and diagnosis of cancer. Prognostics markers are indicative of specific outcomes of the disease and can be used to define predictive models that allow the clinicians to predict the likely prognosis of the disease at time of diagnosis. Moreover, prognosis markers are helpful to monitor the patient response to a drug therapy and facilitate a more personalized patient management. A decrease or return to a normal level may indicate that the cancer is responding to therapy, whereas an increase may indicate that the cancer is not responding. After treatment has ended, tumor marker levels may be used to check for
recurrence of the tumor. Finally, therapeutic markers can be used to develop tumor-specific drugs or affinity ligand (i.e. antibodies) for tumor treatment.

Currently, although an abnormal tumor marker level may suggest cancer, this alone is usually not enough to accurately diagnose cancer and their measurement in body fluids is frequently combined with other tests, such as a biopsy and radioscopic examination. Frequently, tumor marker levels are not altered in all of people with a certain cancer disease, especially if the cancer is at early stage. Some tumor marker levels can also be altered in patients with noncancerous conditions. Most biomarkers commonly used in clinical practice do not reach a sufficiently high level of specificity and sensitivity to unambiguously distinguish a tumor from a normal state.

To date the number of markers that are expressed abnormally is limited to certain types/subtypes of cancer, some of which are also found in other diseases. (http://www.cancer.gov/cancertopics/factsheet).

For example, prostate-specific antigen (PSA) levels are often used to screen men for prostate cancer, but this is controversial since elevated PSA levels can be caused by both prostate cancer or benign conditions, and most men with elevated PSA levels turn out not to have prostate cancer.

Another tumor marker, Cancer Antigen 125, (CA 125), is sometimes used to screen women who have an increased risk for ovarian cancer. Scientists are studying whether measurement of CA 125, along with other tests and exams, is useful to find ovarian cancer before symptoms develop. So far, CA 125 measurement is not sensitive or specific enough to be used to screen all women for ovarian cancer. Mostly, CA 125 is used to monitor response to treatment and check for recurrence in women with ovarian cancer. Finally, human epidermal growth factor receptor (HER2) is a marker protein overproduced in about 20% of breast cancers, whose expression is typically associated with a more aggressive and recurrent tumors of this class.
Routine screening test for tumor diagnosis

Screening tests are a way of detecting cancer early, before there are any symptoms. For a screening test to be helpful it should have high sensitivity and specificity. Sensitivity refers to the test's ability to identify people who have the disease. Specificity refers to the test's ability to identify people who do not have the disease. Different molecular biology approaches such as analysis of DNA sequencing, small nucleotide polymorphisms, in situ hybridization and whole transcriptional profile analysis have done remarkable progresses to discriminate a tumor state from a normal state and are accelerating the knowledge process in the tumor field. However so far different reasons are delaying their use in the common clinical practice, including the higher analysis complexity and their expensiveness. Other diagnosis tools whose application is increasing in clinics include in situ hybridization and gene sequencing.

Currently, Immuno-HistoChemistry (IHC), a technique that allows the detection of proteins expressed in tissues and cells using specific antibodies, is the most commonly used method for the clinical diagnosis of tumor samples. This technique enables the analysis of cell morphology and the classification of tissue samples on the basis of their immunoreactivity. However, at present, IHC can be used in clinical practice to detect cancerous cells of tumor types for which protein markers and specific antibodies are available. In this context, the identification of a large panel of markers for the most frequent cancer classes would have a great impact in the clinical diagnosis of the disease.

Anti-cancer therapies

In the last decades, an overwhelming number of studies remarkably contributed to the comprehension of the molecular mechanisms leading to cancer. However, this scientific progress in the molecular oncology field has
not been paralleled by a comparable progress in cancer diagnosis and therapy. Surgery and/or radiotherapy are still the main modality of local treatment of cancer in the majority of patients. However, these treatments are effective only at initial phases of the disease and in particular for solid tumors of epithelial origin, as is the case of colon, lung, breast, prostate and others, while they are not effective for distant recurrence of the disease. In some tumor classes, chemotherapeutic treatments have been developed, which generally relies on drugs, hormones and antibodies, targeting specific biological processes used by cancers to grow and spread. However, so far many cancer therapies had limited efficacy due to severity of side effects and overall toxicity. Indeed, a major effort in cancer therapy is the development of treatments able to target specifically tumor cells causing limited damages to surrounding normal cells thereby decreasing adverse side effects. Recent developments in cancer therapy in this direction are encouraging, indicating that in some cases a cancer specific therapy is feasible. In particular, the development and commercialization of humanized monoclonal antibodies that recognize specifically tumor-associated markers and promote the elimination of cancer is one of the most promising solution that appears to be an extremely favorable market opportunity for pharmaceutical companies.

However, at present the number of therapeutic antibodies available on the market or under clinical studies is very limited and restricted to specific cancer classes. So far licensed monoclonal antibodies currently used in clinics for the therapy of specific tumor classes show only a partial efficacy and are frequently associated with chemotherapies to increase their therapeutic effect. Administration of Trastuzumab (Herceptin), a commercial monoclonal antibody targeting HER2 in conjunction with Taxol adjuvant chemotherapy induces tumor remission in about 42% of the cases (1). Bevacizumab (Avastin) and Cetuximab (Erbitux) are two monoclonal antibodies recently
licensed for use in humans targeting the endothelial and epithelial growth factors respectively that, combined with adjuvant chemotherapy, proved to be effective against different tumor diseases. Bevacizumab proved to be effective in prolonging the life of patients with metastatic colorectal, breast and lung cancers. Cetuximab demonstrated efficacy in patients with tumor types refractory to standard chemotherapeutic treatments (1).

In summary, available screening tests for tumor diagnosis are uncomfortable or invasive and this sometimes limits their applications. Moreover tumor markers available today have a limited utility in clinics due to either their incapability to detect all tumor subtypes of the defined cancers types and/or to distinguish unambiguously tumor vs. normal tissues. Similarly, licensed monoclonal antibodies combined with standard chemotherapies are not effective against the majority of cases. Therefore, there is a great demand for new tools to advance the diagnosis and treatment of cancer.

**Experimental approaches commonly used to identify tumor markers**

Most popular approaches used to discover new tumor markers are based on genome-wide transcription profile or total protein content analyses of tumor. These studies usually lead to the identification of groups of mRNAs and proteins which are differentially expressed in tumors. Validation experiments then follow to eventually single out, among the hundreds of RNAs/proteins identified, the very few that have the potential to become useful markers. Although often successful, these approaches have several limitations and often, do not provide firm indications on the association of protein markers with tumor. A first limitation is that, since frequently mRNA levels not always correlate with corresponding protein abundance (approx. 50% correlation), studies based on transcription profile do not provide solid information regarding the expression of protein markers in tumor (2, 3, 4, 5).

A second limitation is that neither transcription profiles nor analysis of
total protein content discriminate post-translation modifications, which often occur during oncogenesis. These modifications, including phosphorylations, acetylations, and glycosylations, or protein cleavages influence significantly protein stability, localization, interactions, and functions (6).

As a consequence, large scale studies generally result in long lists of differentially expressed genes that would require complex experimental paths in order to validate the potential markers. However, large scale genomic/proteomic studies reporting novel tumor markers frequently lack of confirmation data on the reported proteins and thus do not provide solid demonstration on the association of the described protein markers with tumor.

**Approach used to identify the protein markers included in the present invention**

The approach that we used to identify protein markers is based on an innovative immuno-proteomic technology. In essence, a library of recombinant human proteins has been produced from E. coli and is being used to generate polyclonal antibodies against each of the recombinant proteins.

The screening of the antibodies library on Tissue microarrays (TMAs), carrying clinical samples from different patients affected by the tumor under investigation, leads to the identification of specific tumor marker proteins. Therefore, by screening TMAs with the antibody library, the tumor markers are visualized by immuno-histochemistry, the classical technology applied in all clinical pathology laboratories. Since TMAs also include healthy tissues, the specificity of the antibodies for the tumors can be immediately appreciated and information on the relative level of expression and cellular localization of the markers could be obtained. In our approach the markers are subjected to a validation process consisting in a molecular and cellular characterization.

Altogether, the detection of the marker proteins disclosed in the present invention selectively in tumor samples and the subsequent validation
experiments leads to an unambiguous confirmation of the marker identity and confirm its association with defined tumor classes. Moreover this experimental process provides an indication of the possible use of the proteins as tools for diagnostic or therapeutic intervention. For instance, proteins showing a cell surface localization could be both diagnostic and therapeutic markers, against which both chemical and antibody therapies can be developed. Differently, markers showing a cytoplasmic localization could be more likely considered for the development of tumor diagnostic tests and chemotherapy/small molecules treatments.

**Summary of the invention**

The present invention provides new means for the detection and treatment of tumors, in particular colo-rectal, lung, prostate and breast cancers, based on the identification of protein markers specific for these tumor types, namely: a) Collectin-1 l (COLECI1) protein, b) Follistatin-like protein 5 (FSTL5) and c) FAM82C (FAM82A2) protein.

In preferred embodiments, the invention provides the use of a) COLECI1 protein as a marker or target for colon tumor, b) FSTL5 protein as a marker or target for colon and prostate tumors, c) FAM82A2 as a marker or target for breast, colon and lung tumors.

The invention also provides a method for the diagnosis of these cancer types, comprising a step of detecting the above-identified markers in a biological sample, e.g. in a tissue sample of a subject suspected of having or at risk of developing malignancies or susceptible to cancer recurrences. In particular, the protein markers of the invention allow to specifically detect lung, colon, breast and prostate cancers, according to their tumor-specificticy, namely: a) COLECI1 protein for colon tumor; b) FSTL5 protein for colon and prostate tumors; c) FAM82A2 protein for breast, colon and lung tumors.

In addition, the tumor markers identify novel targets for affinity ligands...
which can be used for therapeutic applications, especially in the treatment of colon, lung, prostate and breast proliferative diseases. Also provided are affinity ligands, particularly antibodies, capable of selectively interacting with the newly identified protein markers.

**Detailed disclosure of the invention**

The present invention is based on the surprising finding of antibodies that are able to specifically bind tumor tissues from patients, while negative or very poor binding is observed in normal tissues from the same patients. These antibodies have been found to specifically bind to proteins for which no previous association with tumor has been reported. Hence, in a first aspect, the invention provides a tumor marker which is selected from the group consisting of:

a) Collectin-11 in one of its variant isoforms [SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12] or a different isoform having sequence identity of at least 80%, preferably at least 90%, more preferably at least 95% to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 or SEQ ID NO:12; or a nucleic acid molecule containing a sequence coding for a Collectin-11 protein, said encoding sequence being preferably selected from [SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30 and SEQ ID NO:31];

b) FSTL5 protein in one of its variant isoforms [SEQ ID NO:13] or SEQ
ID NO:14, or a different isoform having sequence identity of at least 80%, preferably at least 90%, more preferably at least 95% to SEQ ID NO: 13 or SEQ ID NO: 14; or a nucleic acid molecule containing a sequence coding for a FSTL5 protein, said encoding sequence being preferably selected from SEQ ID NO: 32, SEQ ID NO: 33;

c) FAM82A2 protein in one of its variant isoforms SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 17, or a different isoform having sequence identity of at least 80%, preferably at least 90%, more preferably at least 95% to SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 17; or a nucleic acid molecule containing a sequence coding for a FAM82A2 protein, said encoding sequence being preferably selected from SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36;

Collectin-I 1 (Gene names: COLECI 1, Gene ID: ENSGO000118004, Protein name: Collectin-I 1, Alternative name(s): Collectin kidney protein 1, CL-Kl, ORF Names: UNQ596/PRO1 182, Protein ID: ENSP00000384882, ENSP00000339168, ENSP00000236693, ENSP00000371494, ENSP00000385130, ENSP00000385653, ENSP00000385827, Transcript ID: ENST00000402794, ENST00000439077, ENST00000236693, ENST00000382062, ENST00000403096, ENST00000402922, ENST00000404205) is a protein without previous known association with tumor classes under investigation and is preferably used as a marker for colon tumor and in general for cancers of these types. As described below, an antibody generated towards the COLECI 1 protein shows a selective immunoreactivity in histological preparation of colo-rectal cancer tissues, which indicates the presence of COLECI 1 in these cancer samples and makes COLECI 1 protein and its antibody highly interesting tools for specifically distinguishing a colo-rectal cancer from a normal state.
Follistatin-related protein 5 (Gene name: FSTL5, Synonyms: KIAA1263, Gene ID: ENSG00000168843, Protein name(s): Follistatin-related protein 5, Follistatin-like 5, Protein ID: ENSP00000368462, ENSP00000305334, Transcript ID: ENST00000379164, ENST00000306100) is a protein without previous known association with tumor classes under investigation and is preferably used as a marker for colon and prostate tumors, and in general for cancers of these types. As described below, an antibody generated towards a fragment of FSTL5 shows a selective immunoreactivity in histological preparation of colo-rectal cancer tissues and prostate cancer tissues, which indicates the presence of this protein in these cancer samples.

FAM82A2 (Gene ID: ENSG00000137824, Gene Name: FAM82A2, Synonyms: FAM82C, PTPIP51, Protein name: FAM82A2, Regulator of microtubule dynamics protein 3, RMD-3, hRMD-3, FAM82A2, FAM82C, Protein tyrosine phosphatase-interacting protein 51, TCPTP-interacting protein 51, Cerebral protein 10; Protein ID: ENSP00000260385, ENSP00000342493, ENSP000003 80607, Transcript ID: ENST00000260385, ENST00000338376, ENST00000397465) is a protein without previous known association with tumor classes under investigation and is preferably used as a marker for breast, colon and lung tumors, and in general for cancers of these types. As described below, an antibody generated towards a fragment of FAM82A2 shows a selective immunoreactivity in histological preparation of breast cancer tissues, colon cancer tissues and lung cancer tissues, which indicates the presence of this protein in these cancer samples.

A further aspect of this invention is a method of screening a tissue sample for malignancy, which comprises determining the presence in said sample of at least one of the above-mentioned tumor markers. This method includes detecting either the marker protein, e.g. by means of labeled monoclonal or polyclonal antibodies that specifically bind to the target
protein, or the respective mRNA, e.g. by means of polymerase chain reaction techniques such as RT-PCR. The methods for detecting proteins in a tissue sample are known to one skilled in the art and include immunoradiometric, immunoenzymatic or immunohistochemical techniques, such as radioimmunoassays, immunofluorescent assays or enzyme-linked immunoassays. Other known protein analysis techniques, such as polyacrylamide gel electrophoresis (PAGE), Western blot or Dot blot are suitable as well. Preferably, the detection of the protein marker is carried out with the immune-histochemistry technology, particularly by means of High Through-Put methods that allow the analyses of the antibody immune-reactivity simultaneously on different tissue samples immobilized on a microscope slide. Briefly, each Tissue Micro Array (TMA) slide includes tissue samples suspected of malignancy taken from different patients, and an equal number of normal tissue samples from the same patients as controls. The direct comparison of samples by qualitative or quantitative measurement, e.g. by enzimatic or colorimetric reactions, allows the identification of tumors.

In one embodiment, the invention provides a method of screening a sample of colon or colo-rectal tissue for malignancy, which comprises determining the presence in said sample of a tumor marker selected from COLECII, FSTL5 and FAM82A2 proteins, variants or isoforms or combinations thereof as described above. In another embodiment, the invention provides a method of screening a sample of lung tissue for malignancy, which comprises determining the presence in said sample of the FAM82A2 protein tumor marker, variants or isoforms thereof as described above. In a further embodiment, the invention provides a method of screening a sample of breast tissue for malignancy, which comprises determining the presence in said sample of the FAM82A2 protein tumor marker, variants or isoforms thereof as described above. In a yet further embodiment, the
invention provides a method of screening a sample of prostate tissue for malignancy, which comprises determining the presence in said sample of the FSTL5 protein tumor marker, variants or isoforms thereof as described above.

A further aspect of the invention is a method in vitro for determining the presence of a tumor in a subject, which comprises the steps of:

1. providing a sample of the tissue suspected of containing tumor cells;
2. determining the presence of a tumor marker as above defined, or a combination thereof in said tissue sample by detecting the expression of the marker protein or the presence of the respective mRNA transcript;

wherein the detection of one or more tumor markers in the tissue sample is indicative of the presence of tumor in said subject.

The methods and techniques for carrying out the assay are known to one skilled in the art and are preferably based on immunoreactions for detecting proteins and on PCR methods for the detection of mRNAs. The same methods for detecting proteins or mRNAs from a tissue sample as disclosed above can be applied.

A further aspect of this invention is the use of the tumor markers herein provided as targets for the identification of candidate antitumor agents.

Accordingly, the invention provides a method for screening a test compound which comprises contacting the cells expressing a tumor-associated protein selected from Collectin-1, Follistatin-like protein 5 and FAM82A2 with the test compound, and determining the binding of said compound to said cells. In addition, the ability of the test compound to modulate the activity of each target molecule can be assayed.

A further aspect of the invention is a method of suppressing the function or expression of a tumor-associated protein herein provided. This includes inhibiting or blocking the protein, e.g. by means of antibodies, or
silencing the gene encoding therefor, e.g. by RNA interference or RNA antisense technologies. As shown in the experimental section, marker expression confers a malignant phenotype to cells, making them able to grow and proliferate in an anchorage-independent fashion in an in vitro assay.

In one embodiment, the invention provides an antibody or a fragment thereof which is able to specifically recognize and bind to one of the tumor-associated proteins described above. The term "antibody" as used herein refers to all types of immunoglobulins, including IgG, IgM, IgA, IgD and IgE. Such antibodies may include polyclonal, monoclonal, chimeric, single chain, antibodies or fragments such as Fab or scFv. The antibodies may be of various origin, including human, mouse, rat, rabbit and horse, or chimeric antibodies. The production of antibodies is well known in the art. For the production of antibodies in experimental animals, various hosts including goats, rabbits, rats, mice, and others, may be immunized by injection with polypeptides of the present invention or any fragment or oligopeptide or derivative thereof which has immunogenic properties or forms a suitable epitope. Monoclonal antibodies may be produced following the procedures described in Kohler and Milstein, Nature 265:495 (1975) or other techniques known in the art.

The antibodies to the tumor markers of the invention can be used to detect the presence of the marker in histologic preparations or to distinguish tumor cells from normal cells. To that purpose, the antibodies may be labeled with radiocative, fluorescent or enzyme labels.

In addition, the antibodies can be used for treating proliferative diseases by modulating, e.g. inhibiting or abolishing the activity of a target protein according to the invention. Therefore, in a further aspect the invention provides the use of antibodies to a tumor-associated protein selected from Collectin-1, Follistatin-like protein 5 and FAM82A2, for the preparation of a therapeutical agent for the treatment of proliferative diseases. For use in
therapy, the antibodies can be formulated with suitable carriers and excipients, optionally with the addition of adjuvants to enhance their effects.

In a further embodiment, the invention provides a small interfering RNA (siRNAs) complementary to a sequence selected from the group consisting of SEQ ID NO:37 through SEQ ID NO:45, for use in tumor-gene silencing.

A further aspect of the invention relates to a diagnostic kit containing suitable means for detection, in particular the polypeptides or polynucleotides, antibodies or fragments or derivatives thereof described above, reagents, buffers, solutions and materials needed for setting up and carrying out the immunoassays, nucleic acid hybridization or PCR assays described above. Parts of the kit of the invention can be packaged individually in vials or bottles or in combination in containers or multicontainer units.

**Description of the Figures**

**Figure 1.** Analysis of purified Collectin-11 recombinant protein expressed in *E.coli*. Left panel: Comassie staining of purified His-tag-Collectin-11 fusion protein expressed in *E. coli* separated by SDS-PAGE; Right panel: WB on the purified recombinant Collectin-11 protein stained with a specific antibody. Arrow marks the protein band of the expected size. Molecular weight markers are reported on the left.

**Figure 2.** Example images of immuno-histochemistry analysis of tumor (left panels) and normal tissue samples (right panels) stained with anti-Collectin-11 antibodies. The antibody-stains specifically tumor cells (in dark gray); Boxed image represents a zoomed view of the sample section.

**Figure 3.** Collectin-11 expression in transiently transfected HeLa cells. Western blot analysis of Collectin-11 expression in total protein extracts from HeLa cells (corresponding to 1x10^6 cells) transfected with the empty vector pcDNA3 (lane 1) or with the plasmid encoding the COLECI1 gene (lane 2)
stained with a specific antibody. Arrow marks the expected Collectin-1 band. Molecular weight markers are reported on the left.

**Figure 4.** Analysis of purified FSTL5 recombinant protein expressed in *E.coli*. Left panel: Comassie staining of purified His-tag FSTL5 fusion protein expressed in *E. coli* separated by SDS-PAGE; Right panel: WB on the purified recombinant FSTL5 protein stained with an anti-FSTL5 antibody. Arrow marks the protein band of the expected size. Molecular weight markers are reported on the left.

**Figure 5.** Example images of immuno-histochemistry analysis of tumor (left panels) and normal tissue samples (right panels) stained with an anti-FSTL5 antibody. The antibody stains specifically tumor cells (in dark gray); Boxed images represent zoomed views of corresponding sample sections.

**Figure 6.** FSTL5 is secreted by transiently transfected in HeLa cells—Western blot analysis of total protein extracts (lanes 1, 2) or cell culture supernatants (lanes 3, 4) from HeLa cells transfected with the empty vector pcDNA3 (lanes 1, 3) or with the plasmid construct encoding the FSTL5 gene (lanes 2, 4) stained with a specific antibody. Arrow marks the expected FSTL5 band. Molecular weight markers are reported on the left.

**Figure 7.** Analysis of purified FAM82A2 recombinant protein expressed in *E.coli*. Left panel: Comassie staining of purified His-tag FAM82A2 fusion protein expressed in *E. coli* separated by SDS-PAGE; Right panel: WB on the purified recombinant FAM82A2 protein stained with an anti-FAM82A2 antibody. Arrow marks the protein band of the expected size. Additional protein species visible on the gel correspond to FAM82A2 degradation products, as verified by mass spectrometry analysis. Molecular weight markers are reported on the left.

**Figure 8.** Example images of immuno-histochemistry analysis of tumor (left panels) and normal tissue samples (right panels) stained with an anti-
FAM82A2 antibody. The antibody stains specifically tumor cells (in dark gray)

**Figure 9.** FAM82A2 expression in transiently transfected HeLa cells. Western blot analysis of FAM82A2 expression in total protein extracts from HeLa cells (corresponding to 1x10^6 cells) transfected with the empty vector pcDNA3 (lane 1) or with the plasmid construct encoding the FAM82A2 gene (lane 2) stained with a specific antibody. Arrow marks the expected FAM82A2 band. Molecular weight markers are reported on the left.

**Figure 10.** Western blot analysis of FAM82A2 expression in tumor cell lines. Total protein extracts (corresponding to 1x10^6 cells) from the human breast tumor cell lines MDA-MB231 (lane 1) and BT549 (lane 2), lung tumor cell line H-226 (lane 3) were separated by SDS-PAGE, transferred onto nitrocellulose membranes and probed with anti-FAM82A2 antibodies. Arrow marks the expected FAM82A2 band. Molecular weight markers are reported on the left.

**Figure 11.** Confirmation of FAM82A2 antibody specificity upon gene specific silencing. At different time points, total protein extracts from the breast tumor cell line MDA-MB231 untreated (left panel) or transfected with a FAM82A2-specific siRNA (right panel) were separated by SDS-PAGE, transferred onto nitrocellulose membranes and probed with anti-FAM82A2 antibodies. As normalization control, membranes were also probed with an anti-actin antibody.

**Figure 12.** Detection of FAM82A2 in breast tumor tissue homogenates. Examples of breast tissue homogenates of tumor (lanes 3,4) and normal samples (lanes 1, 2) stained with anti-FAM82A2 antibodies. Molecular weight markers are reported on the left. Arrow mark the expected FAM82A2 band.

**Figure 13.** FAM82A2 confers an invasive phenotype to lung tumor cells.

The graph reports the effect of the inhibition of FAM82A2 expression
with a specific siRNA on the migration activity of the H226 lung tumor cell line, measured with the Boyden assay. As controls, cells either untransfected or transfected with a scrambled siRNA were used. Small boxes under the columns show the visual counting of the migrated cells.

EXAMPLES

Example 1. Generation of recombinant human protein antigens and antibodies to identify tumor markers

Methods

The entire coding region or suitable fragments of the genes encoding the target proteins, were designed for cloning and expression using bioinformatic tools with the human genome sequence as template (Lindskog M et al (2005). Where present, the leader sequence for secretion was replaced with the ATG codon to drive the expression of the recombinant proteins in the cytoplasm of E. coli. For cloning, genes were PCR-amplified from cDNA derived from Mammalian Gene Collection (http://mgc.nci.nih.gov/) clones using specific primers so as to fuse a 6 histidine tag sequence at the 3’ end, annealed to in house developed vectors, derivatives of vector pSP73 (Promega) adapted for the T4 ligation independent cloning method (Nucleic Acids Res. 1990 October 25; 18(20): 6069-6074) and used to transform E.coli NovaBlue cells recipient strain. E. coli transformants were plated onto selective LB plates containing 100 μg/ml ampicillin (LB Amp) and positive E.coli clones were identified by restriction enzyme analysis of purified plasmid followed by DNA sequence analysis. For expression, plasmids were used to transform BL21-(DE3) E.coli cells and BL21-(DE3) E. coli cells harbouring the plasmid were inoculated in ZYP-5052 growth medium (Studier, 2005) and grown at 37°C for 24 hours. Afterwards, bacteria were collected by centrifugation, lysed into B-Per Reagent containing 1 mM MgC12, 100 units DNAse I (Sigma), and 1 mg/ml lysozime (Sigma). After 30 min at room
temperature under gentle shaking, the lysate was clarified by centrifugation at
30,000 g for 40 min at 4°C. All proteins were purified from the inclusion
bodies by resuspending the pellet coming from lysate centrifugation in 40 mM
TRIS-HCl, 1 mM TCEP (Tris(2-carboxyethyl)-phosphine hydrochloride,
Pierce) and 6M guanidine hydrochloride, pH 8 and performing an IMAC in
denaturing conditions. Briefly, the resuspended material was clarified by
centrifugation at 30,000 g for 30 min and the supernatant was loaded on 0.5
ml columns of Ni-activated Chelating Sepharose Fast Flow (Pharmacia). The
column was washed with 50 mM TRIS-HCl buffer, 1 mM TCEP, 6M urea, 60
mM imidazole, 0.5M NaCl, pH 8. Recombinant proteins were eluted with the
same buffer containing 500 mM imidazole. Proteins were analysed by
SDS-Page and their concentration was determined by Bradford assay using the
BIORAD reagent (BIORAD) with a bovine serum albumin standard according
to the manufacturer's recommendations.

The identity of recombinant affinity purified proteins was further
confirmed by tandem mass spectrometry (MS/MS), using standard procedures.
This analysis also confirmed that lower mass protein species sometimes
visible on the gels corresponded to truncated forms of the proteins.

To generate antisera, the purified proteins were used to immunize CD1
mice (6 week-old females, Charles River laboratories, 5 mice per group)
intraperitoneally, with 3 protein doses of 20 micrograms each, at 2 week-
interval. Freund's complete adjuvant was used for the first immunization,
while Freund's incomplete adjuvant was used for the two booster doses. Two
weeks after the last immunization animals were bled and sera collected from
each animal was pooled.

Results

Gene fragments of the expected size were successfully isolated by PCR
from specific clones of the Mammalian Gene Collection using primers specific
for each gene. In particular, for the COLEC1 gene, a fragment corresponding to nucleotides 316-1053 of the transcript (SEQ ID ENST00000349077) of and encoding an amino acid region from 26 to 271 (SEQ ID ENSP00000339168) was obtained.

For the FSTL5 gene, a fragment corresponding to nucleotides 503-1225 of the transcript (SEQ ID ENST00000306100) of and encoding an amino acid region from 23 to 263 (SEQ ID ENSP0000035334) was obtained.

For the FAM82A2, a fragment corresponding to nucleotides 94-1410 of the transcript (SEQ ID ENST00000260385) of and encoding an amino acid region from 32 to 470 (SEQ ID ENSP00000260385) was obtained.

A clone encoding the correct amino acid sequence was identified for each gene/gene fragment and, upon expression in *E. coli*, a protein of the correct size was produced and subsequently purified using affinity chromatography (Figures 1, 4, 7, left panel). Antibodies generated by immunization specifically recognized their target proteins in Western blot (WB) (Figures 1, 4, 7, right panel).

**Example 2. Tissue profiling by immune-histochemistry**

**Methods**

The analysis of the antibodies capability to recognize their target proteins in tumor samples was carried out by Tissue Micro Array (TMA), a miniaturized immuno-histochemistry technology suitable for HTP analysis that allows to analyse the antibody immuno-reactivity simultaneously on different tissue samples immobilized on a microscope slide.

A tissue microarray was prepared containing 100 formalin-fixed paraffin-embedded cores of human tissues from patients affected by colorectal cancer, ovarian cancer, breast cancer, lung cancer, prostate cancer and corresponding normal tissues and analyzed using the specific antibody sample. Briefly, each TMA slide included tumor tissue samples representative of
different well pedigreed patients, representing the 5 cancer types, and an equal number of normal tissue samples from the same patients as controls. In total, the TMA design consisted in 10 tumor samples per each tumor class and 10 normal tissue from 5 well pedigreed patients (equal to two tumor samples and 2 normal tissues from each patient) to identify promising target molecules differentially expressed in cancer and normal tissues. The direct comparison between tumor and normal tissues of each patient allowed the identification of antibodies that stain tumor cells and provide indication of target expression in the tumor under investigation.

All formalin fixed, paraffin embedded tissues used as donor blocks for TMA production were selected from the archives at the IEO (Istituto Europeo Oncologico, Milan). Corresponding whole tissue sections were examined to confirm diagnosis and tumour classification, and to select representative areas in donor blocks. Normal tissues were defined as microscopically normal (non-neoplastic) and were generally selected from specimens collected from the vicinity of surgically removed tumors. The TMA production was performed essentially as previously described (7, 8). Briefly, a hole was made in the recipient TMA block. A cylindrical core tissue sample (1 mm in diameter) from the donor block was acquired and deposited in the recipient TMA block. This was repeated in an automated tissue arrayer "Galileo TMA CK 3500" (BioRep - Milan) until a complete TMA design was produced. TMA recipient blocks were baked at 42 °C for 2 h prior to sectioning. The TMA blocks were sectioned with 2-3 μm thickness using a waterfall microtome (Leica), and placed onto poli-L-lysinated glass slides for immunohistochemical analysis.

Automated immunohistochemistry was performed as previously described (Kampf C. et al. 2004 Clin. Proteomics 1:285-300). In brief, the glass slides were incubated for 30' min in 60°C, de-paraffinized in xylene (2 x 15 min) using the Bio-Clear solution (Midway. Scientific, Melbourne, Australia), and
re-hydrated in graded alcohols. For antigen retrieval, slides were immersed 0.01 M Na-citrate buffer, pH 6.0 at 99°C for 30 min. Slides were placed in the Autostainer (R) (DakoCytomation) and endogenous peroxidase was initially blocked with 3% H2O2, for 5 min. Slides were then blocked in Dako Cytomation Wash Buffer containing 5% Bovine serum albumin (BSA) and subsequently incubated with mouse antibodies for 30' (dilution 1:200 in Dako Real™ dilution buffer). After washing with DakoCytomation wash buffer, slides were incubated with the goat anti-mouse peroxidase conjugated Envision(R) for 30 min each at room temperature (DakoCytomation). Finally, diaminobenzidine (DakoCytomation) was used as chromogen and Harris hematoxylin (Sigma-Aldrich) was used for counterstaining. The slides were mounted with Pertex(R) (Histolab).

The staining results have been evaluated by a trained pathologist at the light microscope, and scored according to both the percentage of immunostained cells and the intensity of staining. The individual values and the combined score (from 0 to 300) were recorded in a custom-tailored database. Digital images of the immunocytochemical findings have been taken at a Leica DM LB light microscope, equipped with a Leica DFC289 color camera.

Results

A TMA design was obtained, representing tumor tissue samples from 5 tumor classes (lung, ovary, prostate, breast and colon) and normal tissues, derived from 5 patients for each tumor type. The results from tissue profiling showed that the antibodies specific for the recombinant proteins (see Example 1) are strongly immunoreactive on several cancer tissues, indicating the presence of the target proteins in tumors tissues, while no or poor reactivity was detected in normal tissues. Based on this finding, the detection of target proteins in tissue samples can be associated with the specific tumor/s.
The capability of target-specific antibodies to stain different tumor tissues is summarized in Table 1. Representative examples of microscopic enlargements of tissue samples stained by each antibody are reported in Figures 2, 5, 8.

**TABLE 1: TUMOR MARKERS IDENTIFIED BY TMA**

<table>
<thead>
<tr>
<th>TARGET</th>
<th>BREAST</th>
<th>COLON</th>
<th>LUNG</th>
<th>OVARY</th>
<th>PROSTATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLLEC 11</td>
<td>0/5</td>
<td>2/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>FSTL 5</td>
<td>0/5</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
</tr>
<tr>
<td>FAM82A2</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

The table reports the number of positive tumor samples out of the five screened after staining with the target specific antibodies.

**Example 3**

*Confirmation of the marker association with the tumor/s by expanded TMA analysis*

*Method*

The association of each protein with the indicated tumors was further confirmed on a larger collection of clinical samples. To this aim, a tissue microarray was prepared for each of the five tumor classes containing 100 formalin-fixed paraffin-embedded cores of human tissues from 50 patients (equal to two tissue samples from each patient). The TMAs were stained with the marker-specific antibodies, using the previously reported procedure. The
staining results were evaluated, as above described, by a trained pathologist at the light microscope.

**Results**

Five TMA designs were obtained, for each of the five tumors, representing tissue samples from 50 patients. The results from tissue profiling showed that the antibodies specific for the three recombinant proteins (see Example 1) are strongly immune-reactive on a large percentage of tumor tissues, indicating that the corresponding proteins are selectively detected in the tumor/s. This finding confirms a strong association of the markers with the specific tumor/s.

The capability of target-specific antibodies to stain different tumor tissues is summarized in Table 2, which reports the percentage of positive tumor tissue samples derived from the 50 patients.

In the case of COLEC11 and FLST5, it has to be pointed that, being they secreted proteins, the immune-istochemistry analysis could underestimate their association with the tumor/s.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Breast</th>
<th>Colon</th>
<th>Lung</th>
<th>Ovary</th>
<th>Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colec11</td>
<td>na</td>
<td>16</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>FLST5</td>
<td>na</td>
<td>nd</td>
<td>na</td>
<td>na</td>
<td>35</td>
</tr>
<tr>
<td>FAM82A2</td>
<td>nd</td>
<td>53</td>
<td>75</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

na: not applicable; nd: not determined

**Example 4. Expression of target proteins in transfected mammalian cells**

**Methods**

The specificity of the antibodies for each target proteins was assessed
by Western blot analysis on total protein extracts from eukaryotic cells transiently transfected with plasmid constructs containing the complete sequences of the genes encoding the target proteins.

To this aim, cDNA were generated from pools of total RNA derived from human testis, human placenta, human bone marrow, human fetal brain, in reverse transcription reactions and the entire coding regions (ORF) of COLECI 1, FLST5 and FAM82A2 were PCR-amplified with specific primers pairs. PCR products were cloned into plasmid pcDNA3 (Invitrogen). The resulting plasmids were used for transfection experiments. To this aim HeLa cells were grown in DMEM-10% FCS supplemented with 1 mM Glutamine were transiently transfected with preparation of the plasmids and with the empty vector as negative control using the Lipofectamine-2000 transfection reagent (Invitrogen). After 48 hours, cells were collected, lysed with PBS buffer containing 1% Triton X100 and expression of target protein(s) was assessed by Western blot analysis on total cell extracts (corresponding to 1x10^6 cells) using specific antibodies. For secretion analysis, 24 hours after transfection the cell medium was removed and replaced with fresh serum-free medium. After additional 24 hour-incubation the cell supernatant was collected and used for analysis. Western blot was performed by separation of the protein extracts (corresponding to approximately 2x10^5 cells) or the culture supernatant (corresponding to approximately 2x10^5 cells) on pre-cast SDS-PAGE gradient gels (NuPage 4-12% Bis-Tris gel, Invitrogen) under reducing conditions, followed by electro-transfer to nitrocellulose membranes (Invitrogen) according to the manufacturer's recommendations. The membranes were blocked in blocking buffer composed of 1x PBS-0.1% Tween 20 (PBST) added with 10% dry milk, for 1 h at room temperature, incubated with the antibody diluted 1:2500 in blocking buffer containing 1% dry milk and washed in PBST-1%. The secondary HRP-conjugated antibody
(goat anti-mouse immunoglobulin-HRP, Perkin Elmer) was diluted 1:5000 in blocking buffer and chemiluminescence detection was carried out using a Chemidoc-IT UVP CCD camera (UVP) and the Western lightning'\textsuperscript{nl} chemiluminescence Reagent Plus (Perkin Elmer), according to the manufacturer's protocol.

**Results**

To confirm the antibody specificity, the complete coding sequence/s for each target protein were cloned in a eukaryotic expression vector and used for transient transfection of HeLa cells.

Expression of the three protein was detected by Western blot in total protein extracts from HeLa cells transfected with the plasmids encoding for COLECII, FLST5 and FAM82A2 using their specific antibodies. For each tested protein a band of the expected size was visible in transfected HeLa cells, while the same band was not visible in HeLa cells transfected with the empty pcDNA3 plasmid. The antibodies recognized specifically their target proteins, since almost a unique single protein band was detected.

Results are represented in Figures 3, 6 and 9.

**Example 5. Expression of target proteins in tumor cell lines**

Expression of target proteins showing positivity by TMA was also assessed by WB on total extracts from a panel of epithelial cell lines derived from the same tumor types. In each analysis, cells were cultured in under ATCC recommended conditions, and sub-confluent cell mono-layers were detached with PBS-0.5 mM EDTA and lysed by several freeze-thaw passages in PBS-1\% Triton. Total protein extracts were loaded on SDS-PAGE (corresponding to approximately 2x10\textsuperscript{5} cells/lane), and subjected to WB with specific antibodies as described above.

**Results**

Expression of the target proteins was verified in a panel of tumor cell
As example, data are shown for the expression of FAM82A2 in a subset of cell lines including BT549 (breast ductal carcinoma), MDA-MB231 (human breast adenocarcinoma) and H226 (lung, squamous cell carcinoma).

FAM82A2 expression was detected in total protein extracts of tested tumor cell lines by immunoblot, confirming its expression in tumor cells derived from the different tumor types. Results are reported in Figure 10.

Example 6. **Confirmation of the specificity of the tumor-reactive antibodies by gene silencing experiments**

**Methods**

The specificity of the polyclonal antibodies for their targets was also confirmed by transient RNA-interference experiments, measuring the loss of detection of the expected protein bands in cell lines upon silencing. For each gene, a set of small interfering RNAs (siRNAs) and controls were obtained from QIAGEN, whose target sequence is reported in Table 3.

### Table 3

<table>
<thead>
<tr>
<th>NCBI gene</th>
<th>mRNA Accessions</th>
<th>siRNA Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colec11</td>
<td>NM_024027, NM_199235</td>
<td>TAGTGCAGTAGTTAAGTCCAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATGGCCTATGCTTAAGGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLST 5</td>
<td>NM_001128427, NM_001128428, NM_020116</td>
<td>TTCATGTAAGTCTGTCGCATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCTAATGAGATTGCGACATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGGTCATGGTGAGCGGTGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGACGACTCAATAAATAAAA</td>
</tr>
<tr>
<td>FAM82A2</td>
<td>NM_018145</td>
<td>CTGCTACAGCCTTGCTTGAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TACCTTACCTACAAAGGTAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAGCGGGACTCTGCACAAAAGGA</td>
</tr>
</tbody>
</table>

The expression of marker genes was knocked down in a panel of epithelial tumor cell lines using marker-specific siRNAs with the HiPerfect transfection reagent (QIAGEN) following the manufacturer's protocol. As
control, cells treated with irrelevant siRNA (scrambled siRNA) were analysed in parallel. At different time points (ranging from 24 to 72 hours) post transfection, we first assessed the reduction of gene transcription by quantitative RT-PCR (Q-RT-PCR) on total RNA, by evaluating the relative marker transcript level, using the beta-actin, GAPDH or MAPK genes as internal normalization control. Afterwards, the loss of protein expression was also confirmed on total protein extracts prepared from the siRNA-treated cell lines by immunoblot, using the same antibodies giving positive immune-staining on tumor tissues. Blots were also probed with an anti-actin antibody as internal normalization control.

**Results**

Examples of this analysis are reported for FAM82A2 in the breast tumor cell line MDA-MB231. Gene silencing experiments with FAM82A2-specific siRNA reduced the marker transcripts (approximately 30-40 fold reduction), as determined by Q-RT-PCR. Under this condition, immunoblot analysis with the marker-specific antibody revealed that a protein band of expected size, visible the in the untreated cell line, was significantly reduced upon si-RNA treatment. Results are reported in Figure 11. Similar results were obtained when the FAM82A2 silencing was carried out on the lung tumor cell line H226.

**Examples 7. The tumor-reactive antibodies are able to recognize proteins of expected size in tumor tissue homogenates**

The presence of protein bands corresponding to the marker proteins was also investigated in tissue homogenates of tumor biopsies from patients affected by tumor (selected from the 5 tumor classes). In these assay, tumor and normal tissues from the same patients were analysed in parallel. Homogenates were prepared by mechanic tissue disruption in buffer containing 40 mM TRIS-HCl, 1 mM TCEP {Tris(2-carboxyethyl)-phosphine
hydrochloride, Pierce} and 6M guanidine hydrochloride, pH 8. Western blot was performed by separation of the total protein extracts (20 µg/lane) proteins were detected by specific antibodies.

Results

Tested antibodies specifically recognized protein species on tumor tissues, while the same bands were either not or faintly visible on normal tissues. Example data are represented for proteins FAM82A2 on breast tissue homogenates. As shown in Figure 12, in the case of FAM62A a band of expected size was detected in breast tumor tissues, while no bands were detected in normal tissue homogenates, confirming the presence of the marker proteins in the tumor.

Example 8. The marker proteins confer malignant cell phenotype

To verify that the proteins included in the present invention can be exploited as targets for therapeutic applications, the effect of marker depletion was evaluated in vitro in cellular studies generally used to define the role of newly discovered proteins in tumorigenesis or tumor progression. Marker-specific knock-down and control tumor cell lines were assayed for their migration phenotype in the Boyden in vitro invasion assay. A brief description of the assay is provided below.

The Boyden chamber assay is based on a chamber of two medium-filled compartments separated by a microporous membrane. Cells are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents are present. After an appropriate incubation time, the membrane between the two compartments is fixed and stained, and the number of cells that have migrated to the lower side of the membrane is determined. Therefore, the Boyden chamber-based cell migration assay has also been called filter membrane migration assay, trans-well migration assay, or chemotaxis assay.
Method

Tumor cell lines previously shown to express the tumor markers were treated with any of the marker specific siRNA molecules proved to inhibit marker expression (see Table 3) and then tested in the Boyden *in vitro* invasion assay, as compared to control cell lines treated with a scramble siRNA.

For this assay, a transwell system, equipped with 8-μm pore polyvinylpirrolidone-free polycarbonate filters, was used. The upper sides of the porous polycarbonate filters were coated with 50 μg/cm² of reconstituted Matrigel basement membrane and placed into six-well culture dishes containing complete growth medium. Cells (1x10⁴ cells/well) were loaded into the upper compartment in serum-free growth medium. After 16 h of incubation at 37°C, non invading cells were removed mechanically using cotton swabs, and the microporous membrane was stained with Diff-Quick solution. Chemotaxis was evaluated by counting the cells migrated to the lower surface of the polycarbonate filters (six randomly chosen fields, mean± SD).

Results

An example of the results are reported for FAM82A2, for which we show that the depletion of marker expression significantly impairs the invasive phenotype of the H226 lung tumor cell line (Figure 13). This indicates that the protein is involved in tumor progression.
References


CLAIMS

1. A tumor marker which is selected from the group consisting of:
   a) Collectin-11 protein in one of its variant isoforms SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 or SEQ ID NO:12, or a different isoform having sequence identity of at least 80%, preferably at least 90%, more preferably at least 95% to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 or SEQ ID NO:12; or a nucleic acid molecule containing a sequence coding for a Collectin-11 protein, said encoding sequence being preferably selected from SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30 and SEQ ID NO:31;
   b) FSTL5 protein in one of its variant isoforms SEQ ID NO:13 or SEQ ID NO:14, or a different isoform having sequence identity of at least 80%, preferably at least 90%, more preferably at least 95% to SEQ ID NO:13 or SEQ ID NO:14; or a nucleic acid molecule containing a sequence coding for a FSTL5 protein, said encoding sequence being preferably selected from SEQ ID NO:32 and SEQ ID NO:33;
   c) FAM82A2 protein in one of its variant isoforms SEQ ID NO:15, SEQ ID NO:16 or SEQ ID NO:17, or a different isoform having sequence identity of at least 80%, preferably at least 90%, more preferably at least 95% to SEQ ID NO:15, SEQ ID NO:16 or SEQ ID NO:17; or a nucleic acid molecule containing a sequence coding for a
FAM82A2 protein, said encoding sequence being preferably selected from SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36.

2. A tumor marker according to claim 1, for use in the detection of lung or breast cancer, wherein said tumor marker is FAM82A2.

3. A tumor marker according to claim 1, for use in the detection of colon or colo-rectal cancers, wherein said tumor marker is selected from COLECII protein and FSTL5, or a combination thereof.

4. A tumor marker according to claim 1, for use in the detection of prostate cancer, wherein said tumor marker is FSTL5.

5. A method of screening a tissue sample for malignancy, said method comprising determining the presence in said sample of at least one of the above-mentioned tumor markers.

6. A method according to claim 5, wherein the tissue sample is a sample of colon or colo-rectal tissue, said method comprising determining the presence in said sample of a tumor marker selected from COLECII protein and FSTL5, or a combination thereof.

7. A method according to claim 5, wherein the tissue sample is a sample of lung tissue, said method comprising determining the presence in said sample of the FAM82A2 tumor marker.

8. A method according to claim 5, wherein the tissue sample is a sample of breast tissue, said method comprising determining the presence in said sample of the FAM82A2 tumor marker.

9. A method according to claim 5, wherein the tissue sample is a sample of prostate tissue, said method comprising determining the presence in said sample of the FSTL5 tumor marker.

10. A method according to claim 5, wherein the tumor marker is a protein, said method being based on immunoradiometric, immunoenzymatic or immunohistochemical techniques.
11. A method according to claim 5, wherein the tumor marker is a nucleic acid molecule, said method being based on polymerase chain reaction techniques.

12. A method *in vitro* for determining the presence of a tumor in a subject, which comprises the steps of:

   (1) providing a sample of the tissue suspected of containing tumor cells;

   (2) determining the presence of a tumor marker according to claim 1 or a combination thereof as per claims 2-4 in said tissue sample by detecting the expression of the marker protein or the presence of the respective mRNA transcript;

   wherein the detection of one or more tumor markers in the tissue sample is indicative of the presence of tumor in said subject.

13. A method of screening a test compound as an antitumor candidate, which comprises contacting cells expressing a tumor marker protein according to claim 1 with the test compound, and determining the binding of said compound to said cells.

14. An antibody or a fragment thereof which is able to specifically recognize and bind to one of the tumor marker proteins according to claim 1.

15. An antibody according to claim 14, which is either monoclonal or polyclonal.

16. A siRNA having a sequence complementary to one of SEQ ID NOs: 37-45, for use in tumor-gene silencing.

17. The use of an antibody according to claim 14 or 15, or a siRNA according to claim 16, for the preparation of a therapeutical agent for the treatment of proliferative diseases.

18. A diagnostic kit containing an antibody according to claims 14-15 and/or an oligonucleotide complementary to a nucleic acid molecule encoding
a tumor marker according to claim 1, and optionally reagents, buffers, solutions and materials to carry out an immunoassay or a PCR assay.
Figure 1
Figure 2
Figure 3
<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>FSTL5</th>
<th>FSTL5</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4
Figure 5
Figure 11
Figure 13
A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/30 C12Q1/68 G01N33/574

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)
C07K C12Q GOIN

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 practical, search terms used)
EPO-Internal, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>EP 1 498 424 A (HINZMANN BERND DR [DE]; HERMANN KLAUS DR [DE]; HEIDEN ESMERALDA [DE]) 19 January 2005 (2005-01-19) sequence 598</td>
<td>1,5,10-18</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C

See patent family annex

Date of the actual completion of the international search

6 April 2010

Date of mailing of the international search report

02/07/2010

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
Rosin, Oliver
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>WO 00/53755 A (GENENTECH INC [US]; ASHKENAZI AVI J [US]; BAKER KEVIN P [US]; GORDON-DOODARD) 14 September 2000 (2000-09-14) 51</td>
<td>1,5, 10-15, 17,18</td>
</tr>
</tbody>
</table>

Retrieved from the Internet:
URL :http://rstl.royal.societypublishing.org/content/138/55.full.pdf> (retrieved on 2009-03-01)
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos [ ]
   because they relate to subject matter not required to be searched by this Authority, namely [ ]

2. Claims Nos [ ]
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically [ ]

3. Claims Nos [ ]
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a) [ ]

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet [ ]

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims [ ]

2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees [ ]

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos [ ]

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims, it is covered by claims Nos 1, 3, 5, 6, 10-18 (also partially) [ ]

Remark on Protest [ ]

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee [ ]
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation [ ]
- No protest accompanied the payment of additional search fees [ ]
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 3, 5, 6, 10-18 (all partially)
   COLEC11 as marker for the detection of tumors.
   ___

2. claims: 1, 3-6, 9-18 (all partially)
   FSTL5 as marker for the detection of tumors.
   ___

3. claims: 1, 2, 5, 7, 8, 10-18 (all partially)
   FAM82A2 as marker for the detection of tumors.
   ___
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO 2004090547 A</td>
<td>21-10-2004</td>
<td>NONE</td>
<td></td>
</tr>
<tr>
<td>US 2005130193 A1</td>
<td>16-06-2005</td>
<td>NONE</td>
<td></td>
</tr>
</tbody>
</table>