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Despite interdisciplinary approaches and exhaustive use of classical therapeutic procedures, cancers are still among the leading causes of death. More recent therapeutic concepts aim at incorporating the patient's immune system into the overall therapeutic concept by using recombinant tumor vaccines and other specific measures such as antibody therapy. A prerequisite for the success of such a strategy is the recognition of tumor-specific or tumor-associated antigens or epitopes by the patient's immune system whose effector functions are to be interventionally enhanced. Tumor cells biologically differ substantially from their nonmalignant cells of origin. These differences are due to genetic alterations acquired during tumor development and result, inter alia, also in the formation of qualitatively or quantitatively altered molecular structures in the cancer cells. Tumor-associated structures of this kind which are recognized by the specific immune system of the tumor-harboring host are referred to as tumor-associated antigens. The specific recognition of tumor-associated antigens involves cellular and humoral mechanisms which are two functionally interconnected units: $CD4^+$ and $CD8^+$ T lymphocytes recognize the processed antigens presented on the molecules of the MHC (major histocompatibility complex) classes II and I, respectively, while B lymphocytes produce circulating antibody molecules which bind directly to unprocessed antigens. The potential clinical-therapeutical importance of tumor-associated antigens results from the fact that the recognition of antigens on neoplastic cells by the immune system leads to the initiation of cytotoxic effector mechanisms and, in the presence of T helper cells, can cause elimination of the cancer cells (Pardoll, *Nat. Med.* 4:525-31, 1998). Accordingly, a

central aim of tumor immunology is to molecularly define these structures. The molecular nature of these antigens has been enigmatic for a long time. Only after development of appropriate cloning techniques has it been possible to screen cDNA expression libraries of tumors systematically for tumor-associated antigens by analyzing the target structures of cytotoxic T lymphocytes (CTL) (van der Bruggen et al., *Science* 254:1643-7, 1991) or by using circulating autoantibodies (Sahin et al., *Curr. Opin. Immunol.* 9:709-16, 1997) as probes. To this end, cDNA expression libraries were prepared from fresh tumor tissue and recombinantly expressed as proteins in suitable systems. Immunoeffectors isolated from patients, namely CTL clones with tumor-specific lysis patterns, or circulating autoantibodies were utilized for cloning the respective antigens.

In recent years a multiplicity of antigens have been defined in various neoplasias by these approaches. However, the probes utilized for antigen identification in the classical methods illustrated above are immunoeffectors (circulating autoantibodies or CTL clones) from patients usually having already advanced cancer. A number of data indicate that tumors can lead, for example, to tolerization and anergization of T cells and that, during the course of the disease, especially those specificities which could cause effective immune recognition are lost from the immunoeffector repertoire. Current patient studies have not yet produced any solid evidence of a real action of the previously found and utilized tumor-associated antigens. Accordingly, it cannot be ruled out that proteins evoking spontaneous immune responses are the wrong target structures.

It was the object of the present invention to provide target structures for a diagnosis and therapy of cancers.

According to the invention, this object is achieved by the subject matter of the claims.

According to the invention, a strategy for identifying and providing antigens expressed in association with a tumor and the nucleic acids coding therefor was pursued. This strategy is based on the fact that particular genes which are expressed in an organ specific manner, e.g. exclusively in colon, lung or kidney tissue, are reactivated also in tumor cells of the respective organs and moreover in tumor cells of other tissues in an ectopic and forbidden manner. First, data mining produces a list as complete as possible of all known organ-specific genes which are then evaluated for their aberrant activation in different tumors by expression analyses by means of specific RT-PCR. Data mining is a known method of identifying tumor-associated genes. In the conventional strategies, however, transcriptoms of normal tissue libraries are usually subtracted electronically from tumor tissue libraries, with the assumption that the remaining genes are tumor-specific (Schmitt et al., *Nucleic Acids Res.* 27:4251-60, 1999; Vasmatzis et al., *Proc. Natl. Acad. Sci. USA.* 95:300-4, 1998; Scheurle et al., *Cancer Res.* 60:4037-43, 2000).

The concept of the invention, which has proved much more successful, however, is based on utilizing data mining for electronically extracting all organ-specific genes and then evaluating said genes for expression in tumors.

The present teaching thus relates in one aspect to a strategy for identifying tissue-specific genes differentially expressed in tumors. Said strategy combines data mining of public sequence libraries ("*in silico*") with subsequent evaluating laboratory-experimental ("wet bench") studies.

According to the invention, a combined strategy based on two different bioinformatic scripts enabled new tumor genes to be identified. These have previously been classified as being purely organ-specific. The finding that these genes are aberrantly activated in tumor cells allows them to be assigned a substantially new quality with functional implications. These tumor-associated genes and the genetic products encoded thereby were identified and provided independently of an immunogenic action.

The tumor-associated antigens identified have an amino acid sequence encoded by a nucleic acid which is selected from the group consisting of (a) a nucleic acid which comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-8, 41-44, 51-59, 84, 117, 119 and 138, a part or derivative thereof, (b) a nucleic acid which hybridizes with the nucleic acid of (a) under stringent conditions, (c) a nucleic acid which is degenerate with respect to the nucleic acid of (a) or (b), and (d) a nucleic acid which is complementary to the nucleic acid of (a), (b) or (c). In a preferred embodiment, a tumor-associated antigen identified has an amino acid sequence encoded by a nucleic acid which is selected from the group consisting of SEQ ID NOs: 1-8, 41-44, 51-59, 84, 117, 119 and 138. In a further preferred embodiment, a tumor-associated antigen identified comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 9-19, 45-48, 60-66, 85, 90-97, 100-102, 105, 106, 111-116, 118, 120, 123, 124, 135-137, 139, and 142-150, a part or derivative thereof.

The present disclosure generally relates to the use of tumor-associated antigens identified according to the disclosure or of parts or derivatives thereof, of nucleic acids coding therefor or of nucleic acids directed against said coding nucleic acids or of

antibodies directed against the tumor-associated antigens identified or parts or derivatives thereof for therapy and diagnosis. This utilization may relate to individual but also to combinations of two or more of these antigens, functional fragments, nucleic acids, antibodies, etc., in one embodiment also in combination with other tumor-associated genes and antigens for diagnosis, therapy and progress control.

Preferred diseases for a therapy and/or diagnosis are those in which one or more of the tumor-associated antigens identified according to the disclosure are selectively expressed or abnormally expressed.

The disclosure also relates to nucleic acids and genetic products which are expressed in association with a tumor cell.

Furthermore, the present technical teaching relates to genetic products, i.e. nucleic acids and proteins or peptides, which are produced by altered splicing (splice variants) of known genes or altered translation using alternative open reading frames. In this aspect nucleic acids are disclosed which comprise a nucleic acid sequence selected from the group consisting of sequences according to SEQ ID NOs: 3-5 of the sequence listing. Moreover, in this aspect, the disclosed teaching relates to proteins or peptides which comprise an amino acid sequence selected from the group consisting of the sequences according to SEQ ID NOs: 10 and 12-14 of the sequence listing. The disclosed splice variants can be used as targets for diagnosis and therapy of tumor diseases.

In particular, the present teaching relates to the amino acid sequence according to SEQ ID NO: 10 of the sequence listing which is encoded by an alternative open reading frame identified according to the invention and differs from the previously described

protein sequence (SEQ ID NO: 9) in additional 85 amino acids at the N terminus of the protein.

Very different mechanisms may cause splice variants to be produced, for example

- utilization of variable transcription initiation sites
- utilization of additional exons
- complete or incomplete splicing out of single or two or more exons,
- splice regulator sequences altered via mutation (deletion or generation of new donor/acceptor sequences),
- incomplete elimination of intron sequences.

Altered splicing of a gene results in an altered transcript sequence (splice variant). Translation of a splice variant in the region of its altered sequence results in an altered protein which may be distinctly different in the structure and function from the original protein. Tumor-associated splice variants may produce tumor-associated transcripts and tumor-associated proteins/antigens. These may be utilized as molecular markers both for detecting tumor cells and for therapeutic targeting of tumors. Detection of tumor cells, for example in blood, serum, bone marrow, sputum, bronchial lavage, bodily secretions and tissue biopsies, may be carried out according to the invention, for example, after extraction of nucleic acids by PCR amplification with splice variant-specific oligonucleotides. In particular, pairs of primers are suitable as oligonucleotides at least one of which binds to the region of the splice variant which is tumor-associated under stringent conditions. According to the disclosure, oligonucleotides described for this purpose in the examples are suitable, in particular oligonucleotides which have or comprise a sequence selected from SEQ ID NOs: 34-36, 39, 40, and 107-110 of the sequence listing. All sequence-dependent detection

systems are suitable for detection. These are, apart from PCR, for example gene chip/microarray systems, Northern blot, RNAse protection assays (RDA) and others. All detection systems have in common that detection is based on a specific hybridization with at least one splice variant-specific nucleic acid sequence. However, tumor cells may also be detected by antibodies which recognize a specific epitope encoded by the splice variant. Said antibodies may be prepared by using for immunization peptides which are specific for said splice variant. In this aspect, the disclosed teaching relates, in particular, to peptides which have or comprise a sequence selected from SEQ ID NOs: 17-19, 111-115, 120, and 137 of the sequence listing and specific antibodies which are directed thereto.

Tumor cells can also be detected by using antibodies which recognize glycosylation variants which are modified in a tumor specific manner. Suitable for the generation of such antibodies are peptide regions which differ between tumor cells and healthy cells with respect to glycosylation. In this aspect, the disclosure relates, in particular, to peptides which have or comprise a sequence selected from SEQ ID NOs: 17-19, 111-115, 120, 137 and 142-145 of the sequence listing and specific antibodies which are directed thereto. Asparagin is transformed into aspartic acid by endogenous deglycosylation of N coupled sugar residues. According to the invention, the proteins described herein can be modified with respect to their sequences in a tumor specific manner and, thus, have different biochemical and antibody binding properties. In this aspect, the disclosed teaching relates, in particular, to peptides which have or comprise a sequence selected from SEQ ID NOs: 146-150 of the sequence listing and specific antibodies which are directed thereto.

Suitable for immunization are particularly the amino acids whose epitopes are distinctly different from the

variant(s) of the genetic product, which is (are) preferably produced in healthy cells. Detection of the tumor cells with antibodies may be carried out here on a sample isolated from the patient or as imaging with intravenously administered antibodies. In addition to diagnostic usability, splice variants having new or altered epitopes are attractive targets for immunotherapy. The epitopes may be utilized for targeting therapeutically active monoclonal antibodies or T lymphocytes. In passive immunotherapy, antibodies or T lymphocytes which recognize splice variant-specific epitopes are adoptively transferred here. As in the case of other antigens, antibodies may be generated also by using standard technologies (immunization of animals, panning strategies for isolation of recombinant antibodies) with utilization of polypeptides which include these epitopes. Alternatively, it is possible to utilize for immunization nucleic acids coding for oligo- or polypeptides which contain said epitopes. Various techniques for in vitro or in vivo generation of epitope-specific T lymphocytes are known and have been described in detail (for example Kessler JH, et al. 2001, Sahin et al., 1997) and are likewise based on utilizing oligo- or polypeptides which contain the splice variant-specific epitopes or nucleic acids coding for said oligo- or polypeptides. Oligo- or polypeptides which contain the splice variant-specific epitopes or nucleic acids coding for said polypeptides may also be used as pharmaceutically active substances in active immunotherapy (vaccination, vaccine therapy).

Also described presently are proteins which differ in nature and degree of their secondary modifications in normal and tumor tissue (for example Durand & Seta, 2000; Clin. Chem. 46: 795-805; Hakomori, 1996; Cancer Res. 56: 5309-18).

The analysis of protein modifications can be done in

Western blots. In particular, glycosylations which as a rule have a size of several kDa result in a higher overall mass of the target protein which can be separated in an SDS-PAGE. For the detection of specific O- and N-glycosidic bonds protein lysates are incubated with O- or N-glycosylases (according to the instructions of the respective manufactures, for example, PNgase, endoglycosidase F, endoglycosidase H, Roche Diagnostics) prior to denaturation using SDS. Thereafter, a Western blot is performed. If the size of target protein is reduced a specific glycosylation can be detected in this manner following incubation with a glycosidase and thus, also the tumor specificity of a modification can be analyzed. Protein regions which are differentially glycosylated in tumor cells and healthy cells are of particular interest. Such differences in glycosylation, however, have hitherto only been described for a few cell surface proteins (for example, Muc1).

According to the invention, it was possible to detect a differential glycosylation for Claudin-18 in tumors. Gastrointestinal carcinomas, pancreas carcinomas, esophagus tumors, prostate tumors as well as lung tumors have a form of Claudin-18 which is glycosylated at a lower level. Glycosylation in healthy tissues masks protein epitopes of Claudin-18 which are not covered on tumor cells due to lacking glycosylation. Correspondingly it is possible according to the invention to select ligands and antibodies which bind to these domains. Such ligands and antibodies according to the invention do not bind to Claudin-18 on healthy cells since here the epitops are covered due to glycosylation.

As has been described above for protein epitopes which are derived from tumor-associated splice variants it is thus possible to use the differential glycosylation to distinguish normal cells and tumor cells with

diagnostic as well as therapeutic intention.

In one aspect, the disclosure relates to a pharmaceutical composition comprising an agent which recognizes the tumor-associated antigen identified according to the disclosure and which is preferably selective for cells which have expression or abnormal expression of a tumor-associated antigen identified according to the disclosure. In particular embodiments, said agent may cause induction of cell death, reduction in cell growth, damage to the cell membrane or secretion of cytokines and preferably have a tumor-inhibiting activity. In one embodiment, the agent is an antisense nucleic acid which hybridizes selectively with the nucleic acid coding for the tumor-associated antigen. In a further embodiment, the agent is an antibody which binds selectively to the tumor-associated antigen, in particular a complement-activated or toxin conjugated antibody which binds selectively to the tumor-associated antigen. In a further embodiment, the agent comprises two or more agents which each selectively recognize different tumor-associated antigens, at least one of which is a tumor-associated antigen identified according to the disclosure. Recognition needs not be accompanied directly with inhibition of activity or expression of the antigen. In this aspect of the disclosed technical teaching, the antigen selectively limited to tumors preferably serves as a label for recruiting effector mechanisms to this specific location. In a preferred embodiment, the agent is a cytotoxic T lymphocyte which recognizes the antigen on an HLA molecule and lyses the cells labeled in this way. In a further embodiment, the agent is an antibody which binds selectively to the tumor-associated antigen and thus recruits natural or artificial effector mechanisms to said cell. In a further embodiment, the agent is a T helper lymphocyte which enhances effector functions of other cells specifically recognizing said antigen.

In one aspect, the disclosed teaching relates to a pharmaceutical composition comprising an agent which inhibits expression or activity of a tumor-associated antigen identified according to the invention. In a preferred embodiment, the agent is an antisense nucleic acid which hybridizes selectively with the nucleic acid coding for the tumor-associated antigen. In a further embodiment, the agent is an antibody which binds selectively to the tumor-associated antigen. In a further embodiment, the agent comprises two or more agents which each selectively inhibit expression or activity of different tumor-associated antigens, at least one of which is a tumor-associated antigen identified according to the disclosure.

The disclosed teaching furthermore relates to a pharmaceutical composition which comprises an agent which, when administered, selectively increases the amount of complexes between an HLA molecule and a peptide epitope from the tumor-associated antigen identified according to the disclosure. In one embodiment, the agent comprises one or more components selected from the group consisting of (i) the tumor-associated antigen or a part thereof, (ii) a nucleic acid which codes for said tumor-associated antigen or a part thereof, (iii) a host cell which expresses said tumor-associated antigen or a part thereof, and (iv) isolated complexes between peptide epitopes from said tumor-associated antigen and an MHC molecule. In one embodiment, the agent comprises two or more agents which each selectively increase the amount of complexes between MHC molecules and peptide epitopes of different tumor-associated antigens, at least one of which is a tumor-associated antigen identified according to the disclosure.

The disclosed teaching furthermore relates to a pharmaceutical composition which comprises one or more

components selected from the group consisting of (i) a tumor-associated antigen identified according to the disclosure or a part thereof, (ii) a nucleic acid which codes for a tumor-associated antigen identified according to the disclosure or for a part thereof, (iii) an antibody which binds to a tumor-associated antigen identified according to the disclosure or to a part thereof, (iv) an antisense nucleic acid which hybridizes specifically with a nucleic acid coding for a tumor-associated antigen identified according to the disclosure, (v) a host cell which expresses a tumor-associated antigen identified according to the disclosure or a part thereof, and (vi) isolated complexes between a tumor-associated antigen identified according to the disclosure or a part thereof and an HLA molecule.

A nucleic acid coding for a tumor-associated antigen identified according to the disclosure or for a part thereof may be present in the pharmaceutical composition in an expression vector and functionally linked to a promoter.

A host cell present in a pharmaceutical composition may secrete the tumor-associated antigen or the part thereof, express it on the surface or may additionally express an HLA molecule which binds to said tumor-associated antigen or said part thereof. In one embodiment, the host cell expresses the HLA molecule endogenously. In a further embodiment, the host cell expresses the HLA molecule and/or the tumor-associated antigen or the part thereof in a recombinant manner. The host cell is preferably nonproliferative. In a preferred embodiment, the host cell is an antigen-presenting cell, in particular a dendritic cell, a monocyte or a macrophage.

An antibody present in a pharmaceutical composition may be a monoclonal antibody. In further embodiments, the

antibody is a chimeric or humanized antibody, a fragment of a natural antibody or a synthetic antibody, all of which may be produced by combinatory techniques. The antibody may be coupled to a therapeutically or diagnostically useful agent.

An antisense nucleic acid present in a pharmaceutical composition may comprise a sequence of 6-50, in particular 10-30, 15-30 and 20-30, contiguous nucleotides of the nucleic acid coding for the tumor-associated antigen identified according to the disclosure.

In further embodiments, a tumor-associated antigen, provided by a pharmaceutical composition of the disclosure either directly or via expression of a nucleic acid, or a part thereof binds to MHC molecules on the surface of cells, said binding preferably causing a cytolytic response and/or inducing cytokine release.

A pharmaceutical composition may comprise a pharmaceutically compatible carrier and/or an adjuvant. The adjuvant may be selected from saponin, GM-CSF, CpG nucleotides, RNA, a cytokine or a chemokine. A pharmaceutical composition is preferably used for the treatment of a disease characterized by selective expression or abnormal expression of a tumor-associated antigen. In a preferred embodiment, the disease is cancer.

The disclosed teaching furthermore relates to methods of treating, diagnosing and/or monitoring a disease characterized by expression or abnormal expression of one of more tumor-associated antigens. In one embodiment, the treatment comprises administering a pharmaceutical composition.

Preferably, the disease is cancer wherein the term

"cancer" comprises but is not limited to leukemias, seminomas, melanomas, teratomas, gliomas, renal, adrenal, thyroid, intestinal, liver, colon, stomach, gastrointestinal, lymph node, esophageal, colorectal, pancreatic, ear, nose and throat (ENT), breast, prostate, uterus, ovarial and lung cancer and the metastases thereof.

In one aspect, the disclosure relates to a method of diagnosing a disease characterized by expression or abnormal expression of a tumor-associated antigen identified according to the disclosure. The method comprises detection of (i) a nucleic acid which codes for the tumor-associated antigen or of a part thereof and/or (ii) detection of the tumor-associated antigen or of a part thereof, and/or (iii) detection of an antibody to the tumor-associated antigen or to a part thereof and/or (iv) detection of cytotoxic or T helper lymphocytes which are specific for the tumor-associated antigen or for a part thereof in a biological sample isolated from a patient. In particular embodiments, detection comprises (i) contacting the biological sample with an agent which binds specifically to the nucleic acid coding for the tumor-associated antigen or to the part thereof, to said tumor-associated antigen or said part thereof, to the antibody or to cytotoxic or T helper lymphocytes specific for the tumor-associated antigen or parts thereof, and (ii) detecting the formation of a complex between the agent and the nucleic acid or the part thereof, the tumor-associated antigen or the part thereof, the antibody or the cytotoxic or T helper lymphocytes. In one embodiment, the disease is characterized by expression or abnormal expression of two or more different tumor-associated antigens and detection comprises detection of two or more nucleic acids coding for said two or more different tumor-associated antigens or of parts thereof, detection of said two or more different tumor-associated antigens or of parts thereof, detection of

two or more antibodies binding to said two or more different tumor-associated antigens or to parts thereof or detection of two or more cytotoxic or T helper lymphocytes specific for said two or more different tumor-associated antigens. In a further embodiment, the biological sample isolated from the patient is compared to a comparable normal biological sample.

In a further aspect, the disclosed teaching relates to a method for determining regression, course or onset of a disease characterized by expression or abnormal expression of a tumor-associated antigen identified according to the disclosure, which method comprises monitoring a sample from a patient who has said disease or is suspected of falling ill with said disease, with respect to one or more parameters selected from the group consisting of (i) the amount of nucleic acid which codes for the tumor-associated antigen or of a part thereof, (ii) the amount of the tumor-associated antigen or a part thereof, (iii) the amount of antibodies which bind to the tumor-associated antigen or to a part thereof, and (iv) the amount of cytolytic T cells or T helper cells which are specific for a complex between the tumor-associated antigen or a part thereof and an MHC molecule. The method preferably comprises determining the parameter(s) in a first sample at a first point in time and in a further sample at a second point in time and in which the course of the disease is determined by comparing the two samples. In particular embodiments, the disease is characterized by expression or abnormal expression of two or more different tumor-associated antigens and monitoring comprises monitoring (i) the amount of two or more nucleic acids which code for said two or more different tumor-associated antigens or of parts thereof, and/or (ii) the amount of said two or more different tumor-associated antigens or of parts thereof, and/or (iii) the amount of two or more antibodies which bind to said two or more different tumor-associated antigens or to

parts thereof, and/or (iv) the amount of two or more cytolytic T cells or of T helper cells which are specific for complexes between said two or more different tumor-associated antigens or of parts thereof and MHC molecules.

According to the invention, detection of a nucleic acid or of a part thereof or monitoring the amount of a nucleic acid or of a part thereof may be carried out using a polynucleotide probe which hybridizes specifically to said nucleic acid or said part thereof or may be carried out by selective amplification of said nucleic acid or said part thereof. In one embodiment, the polynucleotide probe comprises a sequence of 6-50, in particular 10-30, 15-30 and 20-30, contiguous nucleotides of said nucleic acid.

In particular embodiments, the tumor-associated antigen to be detected or the part thereof is present intracellularly or on the cell surface. According to the invention, detection of a tumor-associated antigen or of a part thereof or monitoring the amount of a tumor-associated antigen or of a part thereof may be carried out using an antibody binding specifically to said tumor-associated antigen or said part thereof.

In further embodiments, the tumor-associated antigen to be detected or the part thereof is present in a complex with an MHC molecule, in particular an HLA molecule.

According to the invention, detection of an antibody or monitoring the amount of antibodies may be carried out using a protein or peptide binding specifically to said antibody.

According to the invention, detection of cytolytic T cells or of T helper cells or monitoring the amount of cytolytic T cells or of T helper cells which are specific for complexes between an antigen or a part

thereof and MHC molecules may be carried out using a cell presenting the complex between said antigen or said part thereof and an MHC molecule.

The polynucleotide probe, the antibody, the protein or peptide or the cell, which is used for detection or monitoring, is preferably labeled in a detectable manner. In particular embodiments, the detectable marker is a radioactive marker or an enzymic marker. T lymphocytes may additionally be detected by detecting their proliferation, their cytokine production, and their cytotoxic activity triggered by specific stimulation with the complex of MHC and tumor-associated antigen or parts thereof. T lymphocytes may also be detected via a recombinant MHC molecule or else a complex of two or more MHC molecules which are loaded with the particular immunogenic fragment of one or more of the tumor-associated antigens and which can identify the specific T lymphocytes by contacting the specific T cell receptor.

In a further aspect, the disclosed teaching relates to a method of treating, diagnosing or monitoring a disease characterized by expression or abnormal expression of a tumor-associated antigen identified according to the disclosure, which method comprises administering an antibody which binds to said tumor-associated antigen or to a part thereof and which is coupled to a therapeutic or diagnostic agent. The antibody may be a monoclonal antibody. In further embodiments, the antibody is a chimeric or humanized antibody or a fragment of a natural antibody.

In certain embodiments, the disclosed methods of diagnosing or monitoring a disease characterized by expression or abnormal expression of a tumor associated antigen identified according to the disclosure are performed with aid of or by means of detecting disseminating tumor cells or tumor metastases.

Disseminating tumor cells can be detected, for example, in blood, serum, bone marrow, sputum, bronchial aspirate and/or bronchial lavage.

The disclosed teaching also relates to a method of treating a patient having a disease characterized by expression or abnormal expression of a tumor-associated antigen identified according to the disclosure, which method comprises (i) removing a sample containing immunoreactive cells from said patient, (ii) contacting said sample with a host cell expressing said tumor-associated antigen or a part thereof, under conditions which favor production of cytolytic T cells against said tumor-associated antigen or a part thereof, and (iii) introducing the cytolytic T cells into the patient in an amount suitable for lysing cells expressing the tumor-associated antigen or a part thereof. The present teaching likewise relates to cloning the T cell receptor of cytolytic T cells against the tumor-associated antigen. Said receptor may be transferred to other T cells which thus receive the desired specificity and, as under (iii), may be introduced into the patient.

In one embodiment, the host cell endogenously expresses an HLA molecule. In a further embodiment, the host cell recombinantly expresses an HLA molecule and/or the tumor-associated antigen or the part thereof. The host cell is preferably nonproliferative. In a preferred embodiment, the host cell is an antigen-presenting cell, in particular a dendritic cell, a monocyte or a macrophage.

In a further aspect, the present disclosure relates to a method of treating a patient having a disease characterized by expression or abnormal expression of a tumor-associated antigen, which method comprises (i) identifying a nucleic acid which codes for a tumor-associated antigen identified according to the

invention and which is expressed by cells associated with said disease, (ii) transfecting a host cell with said nucleic acid or a part thereof, (iii) culturing the transfected host cell for expression of said nucleic acid (this is not obligatory when a high rate of transfection is obtained), and (iv) introducing the host cells or an extract thereof into the patient in an amount suitable for increasing the immune response to the patient's cells associated with the disease. The method may further comprise identifying an MHC molecule presenting the tumor-associated antigen or a part thereof, with the host cell expressing the identified MHC molecule and presenting said tumor-associated antigen or a part thereof. The immune response may comprise a B cell response or a T cell response. Furthermore, a T cell response may comprise production of cytolytic T cells and/or T helper cells which are specific for the host cells presenting the tumor-associated antigen or a part thereof or specific for cells of the patient which express said tumor-associated antigen or a part thereof.

The disclosed teaching also relates to a method of treating a disease characterized by expression or abnormal expression of a tumor-associated antigen identified according to the disclosure, which method comprises (i) identifying cells from the patient which express abnormal amounts of the tumor-associated antigen, (ii) isolating a sample of said cells, (iii) culturing said cells, and (iv) introducing said cells into the patient in an amount suitable for triggering an immune response to the cells.

Preferably, the host cells used according to the disclosure are nonproliferative or are rendered nonproliferative. A disease characterized by expression or abnormal expression of a tumor-associated antigen is in particular cancer.

The present disclosure furthermore relates to a nucleic acid selected from the group consisting of (a) a nucleic acid which comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 3-5, a part or derivative thereof, (b) a nucleic acid which hybridizes with the nucleic acid of (a) under stringent conditions, (c) a nucleic acid which is degenerate with respect to the nucleic acid of (a) or (b), and (d) a nucleic acid which is complementary to the nucleic acid of (a), (b) or (c). The disclosure furthermore relates to a nucleic acid, which codes for a protein or polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 10, 12-14, and 146-150, a part or derivative thereof.

In a further aspect, the disclosed teaching relates to promoter sequences of nucleic acids of the disclosure. These sequences may be functionally linked to another gene, preferably in an expression vector, and thus ensure selective expression of said gene in appropriate cells.

In a further aspect, the invention relates to a recombinant nucleic acid molecule, in particular DNA or RNA molecule, which comprises a nucleic acid of the disclosure.

The present teaching also relates to host cells which contain a nucleic acid of the disclosure or a recombinant nucleic acid molecule comprising a nucleic acid of the disclosure.

The host cell may also comprise a nucleic acid coding for a HLA molecule. In one embodiment, the host cell endogenously expresses the HLA molecule. In a further embodiment, the host cell recombinantly expresses the HLA molecule and/or the nucleic acid of the disclosure or a part thereof. Preferably, the host cell is nonproliferative. In a preferred embodiment, the host

cell is an antigen-presenting cell, in particular a dendritic cell, a monocyte or a macrophage.

In a further embodiment, the disclosed teaching relates to oligonucleotides which hybridize with a nucleic acid identified according to the disclosure and which may be used as genetic probes or as "antisense" molecules. Nucleic acid molecules in the form of oligonucleotide primers or competent probes, which hybridize with a nucleic acid identified according to the disclosure or parts thereof, may be used for finding nucleic acids which are homologous to said nucleic acid identified according to the disclosure. PCR amplification, Southern and Northern hybridization may be employed for finding homologous nucleic acids. Hybridization may be carried out under low stringency, more preferably under medium stringency and most preferably under high stringency conditions. The term "stringent conditions" according to the invention refers to conditions which allow specific hybridization between polynucleotides.

In a further aspect, the disclosure relates to a protein, polypeptide or peptide which is encoded by a nucleic acid selected from the group consisting of (a) a nucleic acid which comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 3-5, a part or derivative thereof, (b) a nucleic acid which hybridizes with the nucleic acid of (a) under stringent conditions, (c) a nucleic acid which is degenerate with respect to the nucleic acid of (a) or (b), and (d) a nucleic acid which is complementary to the nucleic acid of (a), (b) or (c). In a preferred embodiment, the disclosure relates to a protein or polypeptide or peptide which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 10, 12-14, and 146-150, a part or derivative thereof.

In a further aspect, the disclosed teaching relates to an immunogenic fragment of a tumor-associated antigen

identified according to the disclosure. Said fragment preferably binds to a human HLA receptor or to a human antibody. A fragment of the disclosure preferably comprises a sequence of at least 6, in particular at least 8, at least 10, at least 12, at least 15, at least 20, at least 30 or at least 50, amino acids.

In this aspect the disclosure relates, in particular, to a peptide which has or comprises a sequence selected from the group consisting of SEQ ID NOs: 17-19, 90-97, 100-102, 105, 106, 111-116, 120, 123, 124, 135-137, 139, and 142-150, a part or derivative thereof.

In a further aspect, the disclosed teaching relates to an agent which binds to a tumor-associated antigen identified according to the disclosure or to a part thereof. In a preferred embodiment, the agent is an antibody. In further embodiments, the antibody is a chimeric, a humanized antibody or an antibody produced by combinatory techniques or is a fragment of an antibody. Furthermore, the disclosure relates to an antibody which binds selectively to a complex of (i) a tumor-associated antigen identified according to the disclosure or a part thereof and (ii) an MHC molecule to which said tumor-associated antigen identified according to the disclosure or said part thereof binds, with said antibody not binding to (i) or (ii) alone. An antibody may be a monoclonal antibody. In further embodiments, the antibody is a chimeric or humanized antibody or a fragment of a natural antibody.

In particular, the present disclosure relates to such an agent, in particular an antibody, which specifically binds to a peptide which has or comprises a sequence selected from the group consisting of SEQ ID NOs: 17-19, 90-97, 100-102, 105, 106, 111-116, 120, 123, 124, 135-137, 139, and 142-150, a part or derivative thereof.

With respect to claudin-18, the invention also relates to agents, in particular antibodies, which specifically bind to one variant of claudin-18. In one embodiment, the agent, in particular an antibody specifically binds to the variant claudin-18A1 (SEQ ID NO: 118). In another embodiment, the agent, in particular an antibody specifically binds to the variant claudin-18A2 (SEQ ID NO: 16). Such specific antibodies may, for example, be obtained by immunizing using the peptides described in Example 4.

Furthermore, the invention with respect to claudin-18 relates to agents, in particular antibodies, specifically binding to a form of claudin-18A2 having a particular glycosylation pattern. In one embodiment, the agent, in particular an antibody, specifically binds to a form of claudin-18A2 which is not glycosylated at one or more potential glycosylation sites. In another embodiment, the agent, in particular an antibody, specifically binds to a form of claudin-18A2 which is glycosylated at one or more potential glycosylation sites. Preferably, such potential glycosylation site relates to one or more positions selected from the group consisting of the amino acid positions 37, 38, 45, 116, 141, 146 and 205 of claudin-18A2. Furthermore, such a potential glycosylation preferably relates to a N glycosylation.

An agent which is specific for a variant or form of claudin-18, in particular an antibody which is specific for a variant or form of claudin-18, in this respect means that the agent or the antibody binds stronger to the variant or form for which it is specific than to another variant or form. An agent, in particular an antibody, binds stronger to a first variant or form or a first epitope compared to the binding to a second variant or form or a second epitope, if it binds to the first variant or form or to the first epitope with a dissociation constant (K_D) which is lower than the

dissociation constant for the second variant or form or the second epitope. Preferably, the dissociation constant (K_D) for the variant or form or the epitope to which the agent, in particular the antibody, binds specifically is more than 10-fold, preferably more than 20-fold, more preferably more than 50-fold, even more preferably more than 100-fold and, in particular, more than 200-fold, 500-fold or 1000-fold lower than the dissociation constant (K_D) for the variant or form or the epitope to which the agent, in particular an antibody, does not bind specifically. Preferably, an agent, in particular an antibody, does not bind or does not essentially bind to the variant or form or the epitope for which the agent, in particular the antibody, is not specific.

The agents described above, in particular the antibodies and derivatives thereof as described herein, which specifically bind to a variant or a form of claudin-18 may also be used in the compositions and methods of the invention.

The invention furthermore relates to a conjugate between an agent of the disclosure which binds to a tumor-associated antigen identified according to the disclosure or to a part thereof or an antibody of the disclosure and a therapeutic or diagnostic agent. In one embodiment, the therapeutic or diagnostic agent is a toxin.

In a further aspect, the disclosed teaching relates to a kit for detecting expression or abnormal expression of a tumor-associated antigen identified according to the invention, which kit comprises agents for detection (i) of the nucleic acid which codes for the tumor-associated antigen or of a part thereof, (ii) of the tumor-associated antigen or of a part thereof, (iii) of antibodies which bind to the tumor-associated antigen or to a part thereof, and/or (iv) of T cells which are

specific for a complex between the tumor-associated antigen or a part thereof and an MHC molecule. In one embodiment, the agents for detection of the nucleic acid or the part thereof are nucleic acid molecules for selective amplification of said nucleic acid, which comprise, in particular a sequence of 6-50, in particular 10-30, 15-30 and 20-30, contiguous nucleotides of said nucleic acid.

Detailed description of the invention

Genes are described herein which are expressed in tumor cells selectively or aberrantly and which are tumor-associated antigens.

According to the disclosure, these genes and/or their genetic products and/or their derivatives and/or parts are preferred target structures for therapeutic approaches. Conceptionally, said therapeutic approaches may aim at inhibiting the activity of the selectively expressed tumor-associated genetic product. This is useful, if said aberrant respective selective expression is functionally important in tumor pathogenecity and if its ligation is accompanied by selective damage of the corresponding cells. Other therapeutic concepts contemplate tumor-associated antigens as labels which recruit effector mechanisms having cell-damaging potential selectively to tumor cells. Here, the function of the target molecule itself and its role in tumor development are totally irrelevant.

"Derivative" of a nucleic acid means according to the disclosure that single or multiple nucleotide substitutions, deletions and/or additions are present in said nucleic acid. Furthermore, the term "derivative" also comprises chemical derivatization of a nucleic acid on a nucleotide base, on the sugar or on the phosphate. The term "derivative" also comprises

nucleic acids which contain nucleotides and nucleotide analogs not occurring naturally.

According to the disclosure, a nucleic acid is preferably deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Nucleic acids comprise according to the invention genomic DNA, cDNA, mRNA, recombinantly produced and chemically synthesized molecules. According to the disclosure, a nucleic acid may be present as a single-stranded or double-stranded and linear or covalently circularly closed molecule.

The nucleic acids described according to the disclosure have preferably been isolated. The term "isolated nucleic acid" means according to the invention that the nucleic acid was (i) amplified *in vitro*, for example by polymerase chain reaction (PCR), (ii) recombinantly produced by cloning, (iii) purified, for example by cleavage and gel-electrophoretic fractionation, or (iv) synthesized, for example by chemical synthesis. An isolated nucleic acid is a nucleic acid which is available for manipulation by recombinant DNA techniques.

A nucleic acid is "complementary" to another nucleic acid if the two sequences are capable of hybridizing and forming a stable duplex with one another, with hybridization preferably being carried out under conditions which allow specific hybridization between polynucleotides (stringent conditions). Stringent conditions are described, for example, in Molecular Cloning: A Laboratory Manual, J. Sambrook et al., Editors, 2nd Edition, Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York, 1989 or Current Protocols in Molecular Biology, F.M. Ausubel et al., Editors, John Wiley & Sons, Inc., New York and refer, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin,

2.5 mM NaH_2PO_4 (pH 7), 0.5% SDS, 2 mM EDTA). SSC is 0.15 M sodium chloride/0.15 M sodium citrate, pH 7. After hybridization, the membrane to which the DNA has been transferred is washed, for example, in 2 × SSC at room temperature and then in 0.1-0.5 × SSC/0.1 × SDS at temperatures of up to 68°C.

According to the invention, complementary nucleic acids have at least 40%, in particular at least 50%, at least 60%, at least 70%, at least 80%, at least 90% and preferably at least 95%, at least 98% or at least 99%, identical nucleotides.

Nucleic acids coding for tumor-associated antigens may, according to the disclosure, be present alone or in combination with other nucleic acids, in particular heterologous nucleic acids. In preferred embodiments, a nucleic acid is functionally linked to expression control sequences or regulatory sequences which may be homologous or heterologous with respect to said nucleic acid. A coding sequence and a regulatory sequence are "functionally" linked to one another, if they are covalently linked to one another in such a way that expression or transcription of said coding sequence is under the control or under the influence of said regulatory sequence. If the coding sequence is to be translated into a functional protein, then, with a regulatory sequence functionally linked to said coding sequence, induction of said regulatory sequence results in transcription of said coding sequence, without causing a frame shift in the coding sequence or said coding sequence not being capable of being translated into the desired protein or peptide.

The term "expression control sequence" or "regulatory sequence" comprises according to the invention promoters, enhancers and other control elements which regulate expression of a gene. In particular embodiments, the expression control sequences can be

regulated. The exact structure of regulatory sequences may vary as a function of the species or cell type, but generally comprises 5'untranscribed and 5'untranslated sequences which are involved in initiation of transcription and translation, respectively, such as TATA box, capping sequence, CAAT sequence, and the like. More specifically, 5'untranscribed regulatory sequences comprise a promoter region which includes a promoter sequence for transcriptional control of the functionally linked gene. Regulatory sequences may also comprise enhancer sequences or upstream activator sequences.

Thus, on the one hand, the tumor-associated antigens illustrated herein may be combined with any expression control sequences and promoters. On the other hand, however, the promoters of the tumor-associated genetic products illustrated herein may be combined with any other genes. This allows the selective activity of these promoters to be utilized.

According to the invention, a nucleic acid may furthermore be present in combination with another nucleic acid which codes for a polypeptide controlling secretion of the protein or polypeptide encoded by said nucleic acid from a host cell. A nucleic acid may also be present in combination with another nucleic acid which codes for a polypeptide causing the encoded protein or polypeptide to be anchored on the cell membrane of the host cell or compartmentalized into particular organelles of said cell. Similarly, a combination with a nucleic acid is possible which represents a reporter gene or any "tag".

In a preferred embodiment, a recombinant DNA molecule is a vector, where appropriate with a promoter, which controls expression of a nucleic acid, for example a nucleic acid coding for a tumor-associated antigen of the disclosure. The term "vector" is used here in its

most general meaning and comprises any intermediary vehicle for a nucleic acid which enables said nucleic acid, for example, to be introduced into prokaryotic and/or eukaryotic cells and, where appropriate, to be integrated into a genome. Vectors of this kind are preferably replicated and/or expressed in the cells. An intermediary vehicle may be adapted, for example, to the use in electroporation, in bombardment with microprojectiles, in liposomal administration, in the transfer with the aid of agrobacteria or in insertion via DNA or RNA viruses. Vectors comprise plasmids, phagemids or viral genomes.

The nucleic acids coding for a tumor-associated antigen identified according to the disclosure may be used for transfection of host cells. Nucleic acids here mean both recombinant DNA and RNA. Recombinant RNA may be prepared by in-vitro transcription of a DNA template. Furthermore, it may be modified by stabilizing sequences, capping and polyadenylation prior to application.

According to the disclosure, the term "host cell" relates to any cell which can be transformed or transfected with an exogenous nucleic acid. The term "host cells" comprises according to the invention prokaryotic (e.g. *E. coli*) or eukaryotic cells (e.g. dendritic cells, B cells, CHO cells, COS cells, K562 cells, yeast cells and insect cells). Particular preference is given to mammalian cells such as cells from humans, mice, hamsters, pigs, goats, primates. The cells may be derived from a multiplicity of tissue types and comprise primary cells and cell lines. Specific examples comprise keratinocytes, peripheral blood leukocytes, stem cells of the bone marrow and embryonic stem cells. In further embodiments, the host cell is an antigen-presenting cell, in particular a dendritic cell, monocyte or a macrophage. A nucleic acid may be present in the host cell in the form of a

single copy or of two or more copies and, in one embodiment, is expressed in the host cell.

According to the disclosure, the term "expression" is used in its most general meaning and comprises the production of RNA or of RNA and protein. It also comprises partial expression of nucleic acids. Furthermore, expression may be carried out transiently or stably. Preferred expression systems in mammalian cells comprise pcDNA3.1 and pRc/CMV (Invitrogen, Carlsbad, CA), which contain a selectable marker such as a gene imparting resistance to G418 (and thus enabling stably transfected cell lines to be selected) and the enhancer-promoter sequences of cytomegalovirus (CMV).

In those cases of the disclosure in which an HLA molecule presents a tumor-associated antigen or a part thereof, an expression vector may also comprise a nucleic acid sequence coding for said HLA molecule. The nucleic acid sequence coding for the HLA molecule may be present on the same expression vector as the nucleic acid coding for the tumor-associated antigen or the part thereof, or both nucleic acids may be present on different expression vectors. In the latter case, the two expression vectors may be cotransfected into a cell. If a host cell expresses neither the tumor-associated antigen or the part thereof nor the HLA molecule, both nucleic acids coding therefor are transfected into the cell either on the same expression vector or on different expression vectors. If the cell already expresses the HLA molecule, only the nucleic acid sequence coding for the tumor-associated antigen or the part thereof can be transfected into the cell.

The disclosure also comprises kits for amplification of a nucleic acid coding for a tumor-associated antigen. Such kits comprise, for example, a pair of amplification primers which hybridize to the nucleic

acid coding for the tumor-associated antigen. The primers preferably comprise a sequence of 6-50, in particular 10-30, 15-30 and 20-30 contiguous nucleotides of the nucleic acid and are nonoverlapping, in order to avoid the formation of primer dimers. One of the primers will hybridize to one strand of the nucleic acid coding for the tumor-associated antigen, and the other primer will hybridize to the complementary strand in an arrangement which allows amplification of the nucleic acid coding for the tumor-associated antigen.

"Antisense" molecules or "antisense" nucleic acids may be used for regulating, in particular reducing, expression of a nucleic acid. The term "antisense molecule" or "antisense nucleic acid" refers according to the invention to an oligonucleotide which is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide or modified oligodeoxyribonucleotide and which hybridizes under physiological conditions to DNA comprising a particular gene or to mRNA of said gene, thereby inhibiting transcription of said gene and/or translation of said mRNA. According to the disclosure, an "antisense molecule" also comprises a construct which contains a nucleic acid or a part thereof in reverse orientation with respect to its natural promoter. An antisense transcript of a nucleic acid or of a part thereof may form a duplex with the naturally occurring mRNA specifying the enzyme and thus prevent accumulation of or translation of the mRNA into the active enzyme. Another possibility is the use of ribozymes for inactivating a nucleic acid. Preferred antisense oligonucleotides have a sequence of 6-50, in particular 10-30, 15-30 and 20-30, contiguous nucleotides of the target nucleic acid and preferably are fully complementary to the target nucleic acid or to a part thereof.

In preferred embodiments, the antisense oligonucleotide hybridizes with an N-terminal or 5′ upstream site such as a translation initiation site, transcription initiation site or promoter site. In further embodiments, the antisense oligonucleotide hybridizes with a 3′untranslated region or mRNA splicing site.

In one embodiment, an oligonucleotide consists of ribonucleotides, deoxyribonucleotides or a combination thereof, with the 5′ end of one nucleotide and the 3′ end of another nucleotide being linked to one another by a phosphodiester bond. These oligonucleotides may be synthesized in the conventional manner or produced recombinantly.

In preferred embodiments, an oligonucleotide is a "modified" oligonucleotide. Here, the oligonucleotide may be modified in very different ways, without impairing its ability to bind its target, in order to increase, for example, its stability or therapeutic efficacy. According to the invention, the term "modified oligonucleotide" means an oligonucleotide in which (i) at least two of its nucleotides are linked to one another by a synthetic internucleoside bond (i.e. an internucleoside bond which is not a phosphodiester bond) and/or (ii) a chemical group which is usually not found in nucleic acids is covalently linked to the oligonucleotide. Preferred synthetic internucleoside bonds are phosphorothioates, alkyl phosphonates, phosphorodithioates, phosphate esters, alkyl phosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also comprises oligonucleotides having a covalently modified base and/or sugar. "Modified oligonucleotides" comprise, for example, oligonucleotides with sugar residues which are covalently bound to low molecular weight organic groups

other than a hydroxyl group at the 3′ position and a phosphate group at the 5′ position. Modified oligonucleotides may comprise, for example, a 2′-O-alkylated ribose residue or another sugar instead of ribose, such as arabinose.

Preferably, the proteins and polypeptides described according to the disclosure have been isolated. The terms "isolated protein" or "isolated polypeptide" mean that the protein or polypeptide has been separated from its natural environment. An isolated protein or polypeptide may be in an essentially purified state. The term "essentially purified" means that the protein or polypeptide is essentially free of other substances with which it is associated in nature or *in vivo*.

Such proteins and polypeptides may be used, for example, in producing antibodies and in an immunological or diagnostic assay or as therapeutics. Proteins and polypeptides described according to the disclosure may be isolated from biological samples such as tissue or cell homogenates and may also be expressed recombinantly in a multiplicity of pro- or eukaryotic expression systems.

For the purposes of the present invention, "derivatives" of a protein or polypeptide or of an amino acid sequence comprise amino acid insertion variants, amino acid deletion variants and/or amino acid substitution variants.

Amino acid insertion variants comprise amino- and/or carboxy-terminal fusions and also insertions of single or two or more amino acids in a particular amino acid sequence. In the case of amino acid sequence variants having an insertion, one or more amino acid residues are inserted into a particular site in an amino acid sequence, although random insertion with appropriate screening of the resulting product is also possible.

Amino acid deletion variants are characterized by the removal of one or more amino acids from the sequence. Amino acid substitution variants are characterized by at least one residue in the sequence being removed and another residue being inserted in its place. Preference is given to the modifications being in positions in the amino acid sequence which are not conserved between homologous proteins or polypeptides. Preference is given to replacing amino acids with other ones having similar properties such as hydrophobicity, hydrophilicity, electronegativity, volume of the side chain and the like (conservative substitution). Conservative substitutions, for example, relate to the exchange of one amino acid with another amino acid listed below in the same group as the amino acid to be substituted:

1. small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly)
2. negatively charged residues and their amides: Asn, Asp, Glu, Gln
3. positively charged residues: His, Arg, Lys
4. large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys)
5. large aromatic residues: Phe, Tyr, Trp.

Owing to their particular part in protein architecture, three residues are shown in brackets. Gly is the only residue without a side chain and thus imparts flexibility to the chain. Pro has an unusual geometry which greatly restricts the chain. Cys can form a disulfide bridge.

The amino acid variants described above may be readily prepared with the aid of known peptide synthesis techniques such as, for example, by solid phase synthesis (Merrifield, 1964) and similar methods or by recombinant DNA manipulation. Techniques for introducing substitution mutations at predetermined

sites into DNA which has a known or partially known sequence are well known and comprise M13 mutagenesis, for example. The manipulation of DNA sequences for preparing proteins having substitutions, insertions or deletions, is described in detail in Sambrook et al. (1989), for example.

"Derivatives" of proteins, polypeptides or peptides also comprise single or multiple substitutions, deletions and/or additions of any molecules associated with the enzyme, such as carbohydrates, lipids and/or proteins, polypeptides or peptides. The term "derivative" also extends to all functional chemical equivalents of said proteins, polypeptides or peptides.

A part or fragment of a tumor-associated antigen has a functional property of the polypeptide from which it has been derived. Such functional properties comprise the interaction with antibodies, the interaction with other polypeptides or proteins, the selective binding of nucleic acids and an enzymatic activity. A particular property is the ability to form a complex with HLA and, where appropriate, generate an immune response. This immune response may be based on stimulating cytotoxic or T helper cells. A part or fragment of a tumor-associated antigen of the invention preferably comprises a sequence of at least 6, in particular at least 8, at least 10, at least 12, at least 15, at least 20, at least 30 or at least 50, consecutive amino acids of the tumor-associated antigen.

A part or a fragment of a nucleic acid coding for a tumor-associated antigen relates according to the disclosure to the part of the nucleic acid, which codes at least for the tumor-associated antigen and/or for a part or a fragment of said tumor-associated antigen, as defined above.

The isolation and identification of genes coding for tumor-associated antigens also make possible the diagnosis of a disease characterized by expression of one or more tumor-associated antigens. These methods comprise determining one or more nucleic acids which code for a tumor-associated antigen and/or determining the encoded tumor-associated antigens and/or peptides derived therefrom. The nucleic acids may be determined in the conventional manner, including by polymerase chain reaction or hybridization with a labeled probe. Tumor-associated antigens or peptides derived therefrom may be determined by screening patient antisera with respect to recognizing the antigen and/or the peptides. They may also be determined by screening T cells of the patient for specificities for the corresponding tumor-associated antigen.

The present teaching also enables proteins binding to tumor-associated antigens described herein to be isolated, including antibodies and cellular binding partners of said tumor-associated antigens.

Particular embodiments ought to involve providing "dominant negative" polypeptides derived from tumor-associated antigens. A dominant negative polypeptide is an inactive protein variant which, by way of interacting with the cellular machinery, displaces an active protein from its interaction with the cellular machinery or which competes with the active protein, thereby reducing the effect of said active protein. For example, a dominant negative receptor which binds to a ligand but does not generate any signal as response to binding to the ligand can reduce the biological effect of said ligand. Similarly, a dominant negative catalytically inactive kinase which usually interacts with target proteins but does not phosphorylate said target proteins may reduce phosphorylation of said target proteins as response to a cellular signal. Similarly, a dominant negative transcription factor

which binds to a promoter site in the control region of a gene but does not increase transcription of said gene may reduce the effect of a normal transcription factor by occupying promoter binding sites, without increasing transcription.

The result of expression of a dominant negative polypeptide in a cell is a reduction in the function of active proteins. The skilled worker may prepare dominant negative variants of a protein, for example, by conventional mutagenesis methods and by evaluating the dominant negative effect of the variant polypeptide.

The disclosed teaching also comprises substances such as polypeptides which bind to tumor-associated antigens. Such binding substances may be used, for example, in screening assays for detecting tumor-associated antigens and complexes of tumor-associated antigens with their binding partners and in the purification of said tumor-associated antigens and of complexes thereof with their binding partners. Such substances may also be used for inhibiting the activity of tumor-associated antigens, for example by binding to such antigens.

The disclosure therefore comprises binding substances such as, for example, antibodies or antibody fragments, which are capable of selectively binding to tumor-associated antigens. Antibodies comprise polyclonal and monoclonal antibodies which are produced in the conventional manner.

Such antibodies can recognize proteins in the native and/or denaturated state (Anderson et al., J. Immunol. 143: 1899-1904, 1989; Gardsvoll, J. Immunol. Methods 234: 107-116, 2000; Kayyem et al., Eur. J. Biochem. 208: 1-8, 1992; Spiller et al., J. Immunol. Methods 224: 51-60, 1999).

Antisera which contain specific antibodies specifically binding to the target protein can be prepared by various standard processes; see, for example, "Monoclonal Antibodies: A Practical Approach" by Philip Shepherd, Christopher Dean ISBN 0-19-963722-9; "Antibodies: A Laboratory Manual" by Ed Harlow, David Lane, ISBN: 0879693142 and "Using Antibodies: A Laboratory Manual: Portable Protocol NO" by Edward Harlow, David Lane, Ed Harlow ISBN 0879695447. Thereby it is also possible to generate affine and specific antibodies which recognize complex membrane proteins in their native form (Azorsa et al., J. Immunol. Methods 229: 35-48, 1999; Anderson et al., J. Immunol. 143: 1899-1904, 1989; Gardsvoll, J. Immunol. Methods 234: 107-116, 2000). This is in particular relevant for the preparation of antibodies which are to be used therapeutically, but also for many diagnostic applications. In this respect, it is possible to immunize with the whole protein, with extracellular partial sequences as well as with cells which express the target molecule in physiologically folded form.

Monoclonal antibodies are traditionally prepared using the hybridoma technology. (for technical details see: "Monoclonal Antibodies: A Practical Approach" by Philip Shepherd, Christopher Dean ISBN 0-19-963722-9; "Antibodies: A Laboratory Manual" by Ed Harlow, David Lane ISBN: 0879693142; "Using Antibodies: A Laboratory Manual: Portable Protocol NO" by Edward Harlow, David Lane, Ed Harlow ISBN: 0879695447).

It is known that only a small part of an antibody molecule, the paratope, is involved in binding of the antibody to its epitope (cf. Clark, W.R. (1986), *The Experimental Foundations of Modern Immunology*, Wiley & Sons, Inc., New York; Roitt, I. (1991), *Essential Immunology*, 7th Edition, Blackwell Scientific Publications, Oxford). The pFc' and Fc regions are, for

example, effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically removed or which has been produced without the pFc' region, referred to as $F(ab')_2$ fragment, carries both antigen binding sites of a complete antibody. Similarly, an antibody from which the Fc region has been enzymatically removed or which has been produced without said Fc region, referred to as Fab fragment, carries one antigen binding site of an intact antibody molecule. Furthermore, Fab fragments consist of a covalently bound light chain of an antibody and part of the heavy chain of said antibody, referred to as Fd. The Fd fragments are the main determinants of antibody specificity (a single Fd fragment can be associated with up to ten different light chains, without altering the specificity of the antibody) and Fd fragments, when isolated, retain the ability to bind to an epitope.

Located within the antigen-binding part of an antibody are complementary-determining regions (CDRs) which interact directly with the antigen epitope and framework regions (FRs) which maintain the tertiary structure of the paratope. Both the Fd fragment of the heavy chain and the light chain of IgG immunoglobulins contain four framework regions (FR1 to FR4) which are separated in each case by three complementary-determining regions (CDR1 to CDR3). The CDRs and, in particular, the CDR3 regions and, still more particularly, the CDR3 region of the heavy chain are responsible to a large extent for antibody specificity.

Non-CDR regions of a mammalian antibody are known to be able to be replaced by similar regions of antibodies with the same or a different specificity, with the specificity for the epitope of the original antibody being retained. This made possible the development of "humanized" antibodies in which nonhuman CDRs are covalently linked to human FR and/or Fc/pFc' regions to

produce a functional antibody.

This is utilized in the so called "SLAM" technology, wherein B cells from whole blood are isolated and the cells are monocloned. Then, the supernatant of the single B cells is analyzed with respect to its antibody specificity. In contrast to the hybridoma technology the variable region of the antibody gene is amplified using single cell PCR and cloned into a suitable vector. In this way, the provision of monoclonal antibodies is accelerated (de Wildt et al., J. Immunol. Methods 207: 61-67, 1997).

As another example, WO 92/04381 describes the production and use of humanized murine RSV antibodies in which at least part of the murine FR regions have been replaced with FR regions of a human origin. Antibodies of this kind, including fragments of intact antibodies with antigen-binding capability, are often referred to as "chimeric" antibodies.

The disclosure also provides $F(ab')_2$, Fab, Fv, and Fd fragments of antibodies, chimeric antibodies, in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain-CDR3 regions have been replaced with homologous human or nonhuman sequences, chimeric $F(ab')_2$-fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain-CDR3 regions have been replaced with homologous human or nonhuman sequences, chimeric Fab-fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain-CDR3 regions have been replaced with homologous human or nonhuman sequences, and chimeric Fd-fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced with homologous human or nonhuman sequences. The disclosure also comprises "single-chain" antibodies.

The disclosure also comprises polypeptides which bind specifically to tumor-associated antigens. Polypeptide

binding substances of this kind may be provided, for example, by degenerate peptide libraries which may be prepared simply in solution in an immobilized form or as phage-display libraries. It is likewise possible to prepare combinatorial libraries of peptides with one or more amino acids. Libraries of peptoids and nonpeptidic synthetic residues may also be prepared.

Phage display may be particularly effective in identifying binding peptides of the invention. In this connection, for example, a phage library is prepared (using, for example, the M13, fd or lambda phages) which presents inserts of from 4 to about 80 amino acid residues in length. Phages are then selected which carry inserts which bind to the tumor-associated antigen. This process may be repeated via two or more cycles of a reselection of phages binding to the tumor-associated antigen. Repeated rounds result in a concentration of phages carrying particular sequences. An analysis of DNA sequences may be carried out in order to identify the sequences of the expressed polypeptides. The smallest linear portion of the sequence binding to the tumor-associated antigen may be determined. The "two-hybrid system" of yeast may also be used for identifying polypeptides which bind to a tumor-associated antigen. Tumor-associated antigens described according to the invention or fragments thereof may be used for screening peptide libraries, including phage-display libraries, in order to identify and select peptide binding partners of the tumor-associated antigens. Such molecules may be used, for example, for screening assays, purification protocols, for interference with the function of the tumor-associated antigen and for other purposes known to the skilled worker.

The antibodies described above and other binding molecules may be used, for example, for identifying tissue which expresses a tumor-associated antigen.

Antibodies may also be coupled to specific diagnostic substances for displaying cells and tissues expressing tumor-associated antigens. They may also be coupled to therapeutically useful substances. Diagnostic substances comprise, in a nonlimiting manner, barium sulfate, iocetamic acid, iopanoic acid, calcium ipodate, sodium diatrizoate, meglumine diatrizoate, metrizamide, sodium tyropanoate and radio diagnostic, including positron emitters such as fluorine-18 and carbon-11, gamma emitters such as iodine-123, technetium-99m, iodine-131 and indium-111, nuclides for nuclear magnetic resonance, such as fluorine and gadolinium. According to the invention, the term "therapeutically useful substance" means any therapeutic molecule which, as desired, is selectively guided to a cell which expresses one or more tumor-associated antigens, including anticancer agents, radioactive iodine-labeled compounds, toxins, cytostatic or cytolytic drugs, etc. Anticancer agents comprise, for example, aminoglutethimide, azathioprine, bleomycin sulfate, busulfan, carmustine, chlorambucil, cisplatin, cyclophosphamide, cyclosporine, cytarabidine, dacarbazine, dactinomycin, daunorubin, doxorubicin, taxol, etoposide, fluorouracil, interferon-α, lomustine, mercaptopurine, methotrexate, mitotane, procarbazine HCl, thioguanine, vinblastine sulfate and vincristine sulfate. Other anticancer agents are described, for example, in Goodman and Gilman, "The Pharmacological Basis of Therapeutics", 8th Edition, 1990, McGraw-Hill, Inc., in particular Chapter 52 (Antineoplastic Agents (Paul Calabresi and Bruce A. Chabner). Toxins may be proteins such as pokeweed antiviral protein, cholera toxin, pertussis toxin, ricin, gelonin, abrin, diphtheria exotoxin or *Pseudomonas* exotoxin. Toxin residues may also be high energy-emitting radionuclides such as cobalt-60.

The term "patient" means according to the disclosure a human being, a nonhuman primate or another animal, in

particular a mammal such as a cow, horse, pig, sheep, goat, dog, cat or a rodent such as a mouse and rat. In a particularly preferred embodiment, the patient is a human being.

According to the disclosure, the term "disease" refers to any pathological state in which tumor-associated antigens are expressed or abnormally expressed. "Abnormal expression" means according to the disclosure that expression is altered, preferably increased, compared to the state in a healthy individual. An increase in expression refers to an increase by at least 10%, in particular at least 20%, at least 50% or at least 100%. In one embodiment, the tumor-associated antigen is expressed only in tissue of a diseased individual, while expression in a healthy individual is repressed. One example of such a disease is cancer, wherein the term "cancer" according to the invention comprises leukemias, seminomas, melanomas, teratomas, gliomas, kidney cancer, adrenal cancer, thyroid cancer, intestinal cancer, liver cancer, colon cancer, stomach cancer, gastrointestinal cancer, lymph node cancer, esophagus cancer, colorectal cancer, pancreas cancer, ear, nose and throat (ENT) cancer, breast cancer, prostate cancer, cancer of the uterus, ovarian cancer and lung cancer and the matastases thereof.

According to the invention, a biological sample may be a tissue sample and/or a cellular sample and may be obtained in the conventional manner such as by tissue biopsy, including punch biopsy, and by taking blood, bronchial aspirate, sputum, urine, feces or other body fluids, for use in the various methods described herein.

According to the disclosure, the term "immunoreactive cell" means a cell which can mature into an immune cell (such as B cell, T helper cell, or cytolytic T cell) with suitable stimulation. Immunoreactive cells

comprise $CD34^+$ hematopoietic stem cells, immature and mature T cells and immature and mature B cells. If production of cytolytic or T helper cells recognizing a tumor-associated antigen is desired, the immunoreactive cell is contacted with a cell expressing a tumor-associated antigen under conditions which favor production, differentiation and/or selection of cytolytic T cells and of T helper cells. The differentiation of T cell precursors into a cytolytic T cell, when exposed to an antigen, is similar to clonal selection of the immune system.

Some therapeutic methods are based on a reaction of the immune system of a patient, which results in a lysis of antigen-presenting cells such as cancer cells which present one or more tumor-associated antigens. In this connection, for example autologous cytotoxic T lymphocytes specific for a complex of a tumor-associated antigen and an MHC molecule are administered to a patient having a cellular abnormality. The production of such cytotoxic T lymphocytes *in vitro* is known. An example of a method of differentiating T cells can be found in WO-A-9633265. Generally, a sample containing cells such as blood cells is taken from the patient and the cells are contacted with a cell which presents the complex and which can cause propagation of cytotoxic T lymphocytes (e.g. dendritic cells). The target cell may be a transfected cell such as a COS cell. These transfected cells present the desired complex on their surface and, when contacted with cytotoxic T lymphocytes, stimulate propagation of the latter. The clonally expanded autologous cytotoxic T lymphocytes are then administered to the patient.

In another method of selecting antigen-specific cytotoxic T lymphocytes, fluorogenic tetramers of MHC class I molecule/peptide complexes are used for detecting specific clones of cytotoxic T lymphocytes (Altman et al., *Science* 274:94-96, 1996; Dunbar et al.,

Curr. Biol. 8:413-416, 1998). Soluble MHC class I molecules are folded *in vitro* in the presence of β_2 microglobulin and a peptide antigen binding to said class I molecule. The MHC/peptide complexes are purified and then labeled with biotin. Tetramers are formed by mixing the biotinylated peptide-MHC complexes with labeled avidin (e.g. phycoerythrin) in a molar ratio of 4:1. Tetramers are then contacted with cytotoxic T lymphocytes such as peripheral blood or lymph nodes. The tetramers bind to cytotoxic T lymphocytes which recognize the peptide antigen/MHC class I complex. Cells which are bound to the tetramers may be sorted by fluorescence-controlled cell sorting to isolate reactive cytotoxic T lymphocytes. The isolated cytotoxic T lymphocytes may then be propagated *in vitro*.

In a therapeutic method referred to as adoptive transfer (Greenberg, *J. Immunol.* 136(5):1917, 1986; Riddel et al., *Science* 257:238, 1992; Lynch et al., *Eur. J. Immunol.* 21:1403-1410, 1991; Kast et al., *Cell* 59:603-614, 1989), cells presenting the desired complex (e.g. dendritic cells) are combined with cytotoxic T lymphocytes of the patient to be treated, resulting in a propagation of specific cytotoxic T lymphocytes. The propagated cytotoxic T lymphocytes are then administered to a patient having a cellular anomaly characterized by particular abnormal cells presenting the specific complex. The cytotoxic T lymphocytes then lyse the abnormal cells, thereby achieving a desired therapeutic effect.

Often, of the T cell repertoire of a patient, only T cells with low affinity for a specific complex of this kind can be propagated, since those with high affinity have been extinguished due to development of tolerance. An alternative here may be a transfer of the T cell receptor itself. For this too, cells presenting the desired complex (e.g. dendritic cells) are combined

with cytotoxic T lymphocytes of healthy individuals or another species (e.g. mouse). This results in propagation of specific cytotoxic T lymphocytes with high affinity if the T lymphocytes are derived from a donor organism which had no previous contact with the specific complex. The high affinity T cell receptor of these propagated specific T lymphocytes is cloned. If the high affinity T cell receptors have been cloned from another species they can be humanized to a different extent. Such T cell receptors are then transduced via gene transfer, for example using retroviral vectors, into T cells of patients, as desired. Adoptive transfer is then carried out using these genetically altered T lymphocytes (Stanislawski et al., Nat Immunol. 2:962-70, 2001; Kessels et al., Nat Immunol. 2:957-61, 2001).

The therapeutic aspects above start out from the fact that at least some of the abnormal cells of the patient present a complex of a tumor-associated antigen and an HLA molecule. Such cells may be identified in a manner known per se. As soon as cells presenting the complex have been identified, they may be combined with a sample from the patient, which contains cytotoxic T lymphocytes. If the cytotoxic T lymphocytes lyse the cells presenting the complex, it can be assumed that a tumor-associated antigen is presented.

Adoptive transfer is not the only form of therapy which can be applied according to the invention. Cytotoxic T lymphocytes may also be generated *in vivo* in a manner known per se. One method uses nonproliferative cells expressing the complex. The cells used here will be those which usually express the complex, such as irradiated tumor cells or cells transfected with one or both genes necessary for presentation of the complex (i.e. the antigenic peptide and the presenting HLA molecule). Various cell types may be used. Furthermore, it is possible to use vectors which carry one or both

of the genes of interest. Particular preference is given to viral or bacterial vectors. For example, nucleic acids coding for a tumor-associated antigen or for a part thereof may be functionally linked to promoter and enhancer sequences which control expression of said tumor-associated antigen or a fragment thereof in particular tissues or cell types. The nucleic acid may be incorporated into an expression vector. Expression vectors may be nonmodified extrachromosomal nucleic acids, plasmids or viral genomes into which exogenous nucleic acids may be inserted. Nucleic acids coding for a tumor-associated antigen may also be inserted into a retroviral genome, thereby enabling the nucleic acid to be integrated into the genome of the target tissue or target cell. In these systems, a microorganism such as vaccinia virus, pox virus, Herpes simplex virus, retrovirus or adenovirus carries the gene of interest and de facto "infects" host cells. Another preferred form is the introduction of the tumor-associated antigen in the form of recombinant RNA which may be introduced into cells by liposomal transfer or by electroporation, for example. The resulting cells present the complex of interest and are recognized by autologous cytotoxic T lymphocytes which then propagate.

A similar effect can be achieved by combining the tumor-associated antigen or a fragment thereof with an adjuvant in order to make incorporation into antigen-presenting cells *in vivo* possible. The tumor-associated antigen or a fragment thereof may be represented as protein, as DNA (e.g. within a vector) or as RNA. The tumor-associated antigen is processed to produce a peptide partner for the HLA molecule, while a fragment thereof may be presented without the need for further processing. The latter is the case in particular, if these can bind to HLA molecules. Preference is given to administration forms in which the complete antigen is processed *in vivo* by a dendritic cell, since this may

also produce T helper cell responses which are needed for an effective immune response (Ossendorp et al., *Immunol Lett.* 74:75-9, 2000; Ossendorp et al., *J. Exp. Med.* 187:693-702, 1998). In general, it is possible to administer an effective amount of the tumor-associated antigen to a patient by intradermal injection, for example. However, injection may also be carried out intranodally into a lymph node (Maloy et al., *Proc Natl Acad Sci USA* 98:3299-303, 2001). It may also be carried out in combination with reagents which facilitate uptake into dendritic cells. Preferred tumor-associated antigens comprise those which react with allogenic cancer antisera or with T cells of many cancer patients. Of particular interest, however, are those against which no spontaneous immune responses pre-exist. Evidently, it is possible to induce against these immune responses which can lyse tumors (Keogh et al., *J. Immunol.* 167:787-96, 2001; Appella et al., *Biomed Pept Proteins Nucleic Acids* 1:177-84, 1995; Wentworth et al., *Mol Immunol.* 32:603-12, 1995).

The pharmaceutical compositions described according to the disclosure may also be used as vaccines for immunization. According to the disclosure, the terms "immunization" or "vaccination" mean an increase in or activation of an immune response to an antigen. It is possible to use animal models for testing an immunizing effect on cancer by using a tumor-associated antigen or a nucleic acid coding therefor. For example, human cancer cells may be introduced into a mouse to generate a tumor, and one or more nucleic acids coding for tumor-associated antigens may be administered. The effect on the cancer cells (for example reduction in tumor size) may be measured as a measure for the effectiveness of an immunization by the nucleic acid.

As part of the composition for an immunization, one or more tumor-associated antigens or stimulating fragments thereof are administered together with one or more

adjuvants for inducing an immune response or for increasing an immune response. An adjuvant is a substance which is incorporated into the antigen or administered together with the latter and which enhances the immune response. Adjuvants may enhance the immune response by providing an antigen reservoir (extracellularly or in macrophages), activating macrophages and/or stimulating particular lymphocytes. Adjuvants are known and comprise in a nonlimiting way monophosphoryl lipid A (MPL, SmithKline Beecham), saponins such as QS21 (SmithKline Beecham), DQS21 (SmithKline Beecham; WO 96/33739), QS7, QS17, QS18 and QS-L1 (So et al., Mol. Cells 7:178-186, 1997), incomplete Freund's adjuvant, complete Freund's adjuvant, vitamin E, montanide, alum, CpG oligonucleotides (cf. Kreig et al., Nature 374:546-9, 1995) and various water-in-oil emulsions prepared from biologically degradable oils such as squalene and/or tocopherol. Preferably, the peptides are administered in a mixture with DQS21/MPL. The ratio of DQS21 to MPL is typically about 1:10 to 10:1, preferably about 1:5 to 5:1 and in particular about 1:1. For administration to humans, a vaccine formulation typically contains DQS21 and MPL in a range from about 1 µg to about 100 µg.

Other substances which stimulate an immune response of the patient may also be administered. It is possible, for example, to use cytokines in a vaccination, owing to their regulatory properties on lymphocytes. Such cytokines comprise, for example, interleukin-12 (IL-12) which was shown to increase the protective actions of vaccines (cf. *Science* 268:1432-1434, 1995), GM-CSF and IL-18.

There are a number of compounds which enhance an immune response and which therefore may be used in a vaccination. Said compounds comprise costimulating molecules provided in the form of proteins or nucleic

acids. Examples of such costimulating molecules are B7-1 and B7-2 (CD80 and CD86, respectively) which are expressed on dendritic cells (DC) and interact with the CD28 molecule expressed on the T cells. This interaction provides a costimulation (signal 2) for an antigen/MHC/TCR-stimulated (signal 1) T cell, thereby enhancing propagation of said T cell and the effector function. B7 also interacts with CTLA4 (CD152) on T cells, and studies involving CTLA4 and B7 ligands demonstrate that B7-CTLA4 interaction can enhance antitumor immunity and CTL propagation (Zheng, P. et al., *Proc. Natl. Acad. Sci.* USA 95(11):6284-6289 (1998)).

B7 is typically not expressed on tumor cells so that these are no effective antigen-presenting cells (APCs) for T cells. Induction of B7 expression would enable tumor cells to stimulate more effectively propagation of cytotoxic T lymphocytes and an effector function. Costimulation by a combination of B7/IL-6/IL-12 revealed induction of IFN-gamma and Th1-cytokine profile in a T cell population, resulting in further enhanced T cell activity (Gajewski et al., *J. Immunol.* 154:5637-5648 (1995)).

A complete activation of cytotoxic T lymphocytes and a complete effector function require an involvement of T helper cells via interaction between the CD40 ligand on said T helper cells and the CD40 molecule expressed by dendritic cells (Ridge et al., *Nature* 393:474 (1998), Bennett et al., *Nature* 393:478 (1998), Schönberger et al., *Nature* 393:480 (1998)). The mechanism of this costimulating signal probably relates to the increase in B7 production and associated IL-6/IL-12 production by said dendritic cells (antigen-presenting cells). CD40-CD40L interaction thus complements the interaction of signal 1 (antigen/MHC-TCR) and signal 2 (B7-CD28).

The use of anti-CD40 antibodies for stimulating dendritic cells would be expected to directly enhance a response to tumor antigens which are usually outside the range of an inflammatory response or which are presented by nonprofessional antigen-presenting cells (tumor cells). In these situations, T helper and B7-costimulating signals are not provided. This mechanism could be used in connection with therapies based on antigen-pulsed dendritic cells.

The disclosure also provides for administration of nucleic acids, polypeptides or peptides. Polypeptides and peptides may be administered in a manner known per se. In one embodiment, nucleic acids are administered by *ex vivo* methods, i.e. by removing cells from a patient, genetic modification of said cells in order to incorporate a tumor-associated antigen and reintroduction of the altered cells into the patient. This generally comprises introducing a functional copy of a gene into the cells of a patient *in vitro* and reintroducing the genetically altered cells into the patient. The functional copy of the gene is under the functional control of regulatory elements which allow the gene to be expressed in the genetically altered cells. Transfection and transduction methods are known to the skilled worker. The disclosure also provides for administering nucleic acids *in vivo* by using vectors such as viruses and target-controlled liposomes.

In a preferred embodiment, a viral vector for administering a nucleic acid coding for a tumor-associated antigen is selected from the group consisting of adenoviruses, adeno-associated viruses, pox viruses, including vaccinia virus and attenuated pox viruses, Semliki Forest virus, retroviruses, Sindbis virus and Ty virus-like particles. Particular preference is given to adenoviruses and retroviruses. The retroviruses are typically replication-deficient (i.e. they are incapable of generating infectious

particles).

Various methods may be used in order to introduce according to the disclosure nucleic acids into cells *in vitro* or *in vivo*. Methods of this kind comprise transfection of nucleic acid $CaPO_4$ precipitates, transfection of nucleic acids associated with DEAE, transfection or infection with the above viruses carrying the nucleic acids of interest, liposome-mediated transfection, and the like. In particular embodiments, preference is given to directing the nucleic acid to particular cells. In such embodiments, a carrier used for administering a nucleic acid to a cell (e.g. a retrovirus or a liposome) may have a bound target control molecule. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell may be incorporated into or attached to the nucleic acid carrier. Preferred antibodies comprise antibodies which bind selectively a tumor-associated antigen. If administration of a nucleic acid via liposomes is desired, proteins binding to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation in order to make target control and/or uptake possible. Such proteins comprise capsid proteins or fragments thereof which are specific for a particular cell type, antibodies to proteins which are internalized, proteins addressing an intracellular site, and the like.

The therapeutic compositions of the disclosure may be administered in pharmaceutically compatible preparations. Such preparations may usually contain pharmaceutically compatible concentrations of salts, buffer substances, preservatives, carriers, supplementing immunity-enhancing substances such as adjuvants, CpG and cytokines and, where appropriate, other therapeutically active compounds.

The therapeutically active compounds may be administered via any conventional route, including by injection or infusion. The administration may be carried out, for example, orally, intravenously, intraperitonealy, intramuscularly, subcutaneously or transdermally. Preferably, antibodies are therapeutically administered by way of a lung aerosol. Antisense nucleic acids are preferably administered by slow intravenous administration.

The compositions are administered in effective amounts. An "effective amount" refers to the amount which achieves a desired reaction or a desired effect alone or together with further doses. In the case of treatment of a particular disease or of a particular condition characterized by expression of one or more tumor-associated antigens, the desired reaction relates to inhibition of the course of the disease. This comprises slowing down the progress of the disease and, in particular, interrupting the progress of the disease. The desired reaction in a treatment of a disease or of a condition may also be delay of the onset or a prevention of the onset of said disease or said condition.

An effective amount of a composition of the invention will depend on the condition to be treated, the severeness of the disease, the individual parameters of the patient, including age, physiological condition, size and weight, the duration of treatment, the type of an accompanying therapy (if present), the specific route of administration and similar factors.

The pharmaceutical compositions are preferably sterile and contain an effective amount of the therapeutically active substance to generate the desired reaction or the desired effect.

The doses administered of the compositions may depend

on various parameters such as the type of administration, the condition of the patient, the desired period of administration, etc. In the case that a reaction in a patient is insufficient with an initial dose, higher doses (or effectively higher doses achieved by a different, more localized route of administration) may be used.

Generally, doses of the tumor-associated antigen of from 1 ng to 1 mg, preferably from 10 ng to 100 µg, are formulated and administered for a treatment or for generating or increasing an immune response. If the administration of nucleic acids (DNA and RNA) coding for tumor-associated antigens is desired, doses of from 1 ng to 0.1 mg are formulated and administered.

The pharmaceutical compositions are generally administered in pharmaceutically compatible amounts and in pharmaceutically compatible compositions. The term "pharmaceutically compatible" refers to a nontoxic material which does not interact with the action of the active component of the pharmaceutical composition. Preparations of this kind may usually contain salts, buffer substances, preservatives, carriers and, where appropriate, other therapeutically active compounds. When used in medicine, the salts should be pharmaceutically compatible. However, salts which are not pharmaceutically compatible may used for preparing pharmaceutically compatible salts and are included in the invention. Pharmacologically and pharmaceutically compatible salts of this kind comprise in a nonlimiting way those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic acids, and the like. Pharmaceutically compatible salts may also be prepared as alkali metal salts or alkaline earth metal salts, such as sodium salts, potassium salts or calcium salts.

A pharmaceutical composition of the disclosure may comprise a pharmaceutically compatible carrier. According to the disclosure, the term "pharmaceutically compatible carrier" refers to one or more compatible solid or liquid fillers, diluents or encapsulating substances, which are suitable for administration to humans. The term "carrier" refers to an organic or inorganic component, of a natural or synthetic nature, in which the active component is combined in order to facilitate application. The components of the pharmaceutical composition are usually such that no interaction occurs which substantially impairs the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffer substances such as acetic acid in a salt, citric acid in a salt, boric acid in a salt and phosphoric acid in a salt.

The pharmaceutical compositions may, where appropriate, also contain suitable preservatives such as benzalkonium chloride, chlorobutanol, parabens and thimerosal.

The pharmaceutical compositions are usually provided in a uniform dosage form and may be prepared in a manner known per se. Pharmaceutical compositions of the disclosure may be in the form of capsules, tablets, lozenges, suspensions, syrups, elixir or in the form of an emulsion, for example.

Compositions suitable for parenteral administration usually comprise a sterile aqueous or nonaqueous preparation of the active compound, which is preferably isotonic to the blood of the recipient. Examples of compatible carriers and solvents are Ringer solution and isotonic sodium chloride solution. In addition, usually sterile, fixed oils are used as solution or suspension medium.

The present invention is described in detail by the figures and examples below, which are used only for illustration purposes and are not meant to be limiting. Owing to the description and the examples, further embodiments are accessible to the skilled worker.

Figures:

Fig. 1. Claudin-18A2.1 expression in stomach and esophagus, as well as stomach and pancreas tumors
RT-PCR analysis with claudin-18A2.1-specific primers (SEQ ID NO: 39, 40) showed according to the invention pronounced claudin-18A2.1 expression in 8/10 stomach tumor biopsies and in 3/6 pancreatic tumor biopsies. Distinct expression was also detected in stomach and esophageal normal tissues. In contrast thereto, no expression was detected in the ovary and in ovarian tumors.

Fig. 2. Diagrammatic depiction of claudin-18 conformations

According to the invention, the claudin-18A2 polypeptide can exist on the cell in two conformations. In conformation 1, the protein is present as membrane molecule having four transmembrane domains (TM) and two separate, extracellularly localized domains. In conformation 2, the two hydrophobic regions in the middle (h-phob) do not exert a transmembrane domain function. Thus, in this conformation, compared to conformation 1, additional peptide regions are located extracellularly. In addition, an additional N glycosylation site results in this conformation at position 116 (thicker arrow). All predicted glycosylation domains are shown in the lower part of the figure. Ex1: extracellular domain 1, Ex2: extracellular domain 2, TM: transmembrane domain, H-phob: extracellular hydrophobic region.

Fig. 3. Quantitative expression of claudin-18, variant A1

Claudin-18A1 is detectable in no normal tissue except lung and stomach tissue. Claudin-18A1 is highly expressed in a large number of tumor tissues. Particularly strong expression is found in gastric tumors, lung tumors, pancreatic tumors and esophageal tumors.

Fig. 4. Quantitative expression of claudin-18, variant A2

Claudin-18A2 is detectable in no normal tissue except stomach tissue. Claudin-18A2 is expressed in a large number of tumor tissues, in particular gastric tumors, lung tumors, pancreatic tumors and esophageal tumors.

Fig. 5. Use of claudin-18A2-specific antibodies (extracellular domain)

A: Staining of claudin-18A2-positive gastric tumor cells (SNU-16, fixed with methanol) with an antibody

which was produced by immunization with a peptide (SEQ ID NO: 17). Membrane staining appears particularly strongly in the cell/cell interaction regions. The protein aggregates in focal membrane regions.

B, C, D: Demonstration of the specificity of the antibody by colocalization analysis in claudin-18A2-GFP-transfected 293T cells. B: GFP fluorescence; C: anti-claudin-18A2; D: superimposition.

Fig. 6. Use of claudin-18A2-specific antibodies (extracellular domain)

Membrane staining of claudin-18A2-positive gastric tumor cells (SNU-16) with an antibody which was produced by immunization with a peptide (SEQ ID NO: 113, N-terminally located extracellular domain). A monoclonal antibody which is directed against E-cadherin was used for counterstaining. A: claudin-18A2 antibody; B: anti-E-cadherin counterstaining; C: superimposition.

Fig. 7. Use of antibodies against the C-terminal extracellular domain of claudin-18

Left figures: Membrane staining of claudin-18A2-positive gastric tumor cells (SNU-16) with an antibody which was produced by immunization with a peptide (SEQ ID NO: 116, C-terminally located extracellular domain). A monoclonal antibody which is directed against E-cadherin was used for counterstaining (right figures).

Fig. 8. Use of claudin-18A1-specific antibodies

Top: Weak to absent staining of gastric tumor cells (SNU-16; claudin18A2 positive) with an antibody which was produced by immunization with a claudin-18A1-specific peptide (SEQ ID NO: 115). A: anti-E-cadherin; B: anti-claudin-18A1; C: superimposition.

Below: Demonstration of the specificity of the antibody by colocalization analysis in claudin-18A1-GFP-transfected 293T cells. A: GFP fluorescence; B: anti-

claudin-18A1; C: superimposition.

Fig. 9. Detection of claudin-18A2 in a Western blot

Western blotting with lysates from various healthy tissues with a claudin-18A2-specific antibody directed against the epitope with SEQ ID NO: 17. 1: Stomach; 2: testis; 3: skin; 4: breast; 5: liver; 6: colon; 7: lung; 8: kidney; 9: lymph node normal tissues.

Fig. 10. Claudin-18A2 Western blotting with samples from stomach and stomach tumors, as well as different tumor cell lines

Lysates from stomach and stomach tumors (A, B) and tumor cell lines (C, D) were blotted and tested using a claudin-18A2-specific antibody against the epitope having SEQ ID NO: 17. Stomach tumors show a less glycosylated form of claudin-18A2. PNGase F treatment of stomach lysates leads to the formation of the low-glycosylated form.

A: 1: stomach normal tissue #A; 2: stomach tumor #A; 3: stomach normal tissue #B; 4: stomach tumor #B

B: 1: stomach normal tissue #A; 2: stomach normal tissue #B; 3: stomach normal tissue #B + PNGase F; 4: stomach tumor #C; 5: stomach tumor #D; 6: stomach tumor #D + PNGase F

C: 1: stomach normal tissue; 2: MDA-MB-231; 3: SK-MEL-37; 4: AGS; 5: SNU-1; 6: SNU-16; 7: EFO27; 8: TOV-112D; 9: OVCAR. Note that the tumor cell lines express the deglycosylated variant of claudin-18A2.

D: Summary table of the Western blot data for a selection of cell lines which have been tested using the claudin-18A2 specific antibody.

Fig. 11. Expression of claudin-18 in lung tumors

Low-glycosylated claudin-18A2 variants were detected in lung tumors in accordance with fig. 30. 1: Stomach normal tissue; 2: stomach tumor; 3-9: lung tumors.

Fig. 12. Immunohistochemical analysis of claudin-18 using claudin-18A2-specific antibodies in normal tissues

In gastric mucosa only differentiated epithelial cells at the orifice as well as at the bottom of the glands are stained. Claudin-18A2 is not detectable in stem cells of the stomach. All other investigated normal tissues also do not express this gene such as, for example, shown for kidney, lung and colon.

Fig. 13. Results of the immune histology using claudin-18A2 specific polyclonal antiserum

A: Examples for specific staining of lung tumor tissues. Note that the normal lung tissue expressing the variant claudin-18A1 is not recognized by the claudin-18A2 specific antiserum.

B: Examples for specific tumor staining of esophageal tumors. Note that healthy cells in the vicinity are not stained.

C: Examples for specific tumor staining of stomach tumor epithelia. Also here healthy cells in the vicinity are not stained.

D: Exemplary summary table of immunohistochemical staining data using claudin-18A2 specific antibodies. AdenoCa: adenocarcinoma; SCC: squamous epithelium carcinoma; RCC: renal cell carcinoma.

Fig. 14. Sequences

The sequences to which reference is made herein are shown.

Fig. 15. Determination of extracellular regions of claudin-18A2

Three constructs were prepared which each had a marker sequence (myc or HA tag) in one of the domains EX1 (= extracellular domain 1), EX2 (= extracellular domain 2) or D3 (= domain 3) (top). These were transfected into cell lines and then tested whether an antibody directed against these marker sequences binds to non-

permeabilized cells. This requires the respective region of the protein to be topologically extracellular. The flow-through cytometry demonstrated that all three regions of the molecule are accessible for the antibody (below).

Fig. 16. Claudin-18A2 membrane topology

According to our data, claudin-18A2 can exist in conformation 2 wherein the inner two hydrophobic domains do not pass through the cell membrane in an integral manner. In this way, larger regions of this molecule are extracellular. Located herein are also glycosylation domains which, according to our data, are glycosylated in stomach normal tissue, but not in tumors. Thus, epitopes emerge which are specific for tumor tissue.

Fig. 17 FACS analysis for determining the extracellular localization of claudin-18.

The figure shows flow-through cytometric analyses with non-permeabilized cells transfected with full-length claudin-18A1, claudin-18A2 and Mock transfected as well as transfected with portions of claudin-18A2. It is shown that the antibodies mAB1 and mAB2 recognize specifically claudin-18A2 (left column) and the extracellular domain 2 (Ex2, third column) on the cell surface, while claudin-18A1 (second column) and the negative control (last column) are negative. The antibody mAB1 in contrast to mAB2 also binds specifically to the extracellular domain 1 (Ex1, fourth column).

Examples:

Material and methods

The terms "*in silico*", "electronic" and "virtual cloning" refer solely to the utilization of methods based on databases, which may also be used to simulate laboratory experimental processes.

Unless expressly defined otherwise, all other terms and expressions are used so as to be understood by the skilled worker. The techniques and methods mentioned are carried out in a manner known per se and are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. All methods including the use of kits and reagents are carried out according to the manufacturers' information.

Datamining-based strategy for determining new tumor-associated genes

Two *in silico* strategies, namely GenBank keyword search and the cDNAxProfiler, were combined. Utilizing the NCBI ENTREZ Search and Retrieval System (http://www.ncbi.nlm.nih.gov/Entrez), a GenBank search was carried out for candidate genes annotated as being specifically expressed in specific tissues (Wheeler et al., *Nucleic Acids Research* 28:10-14, 2000).

Carrying out queries with keywords such as "colon-specific gene", "stomach-specific gene" or "kidney-specific gene", candidate genes (GOI, genes of interest) were extracted from the databases. The search was restricted to part of the total information of these databases by using the limits "homo sapiens", for the organism, and "mRNA", for the type of molecule.

The list of the GOI found was curated by determining different names for the same sequence and eliminating such redundancies.

All candidate genes obtained by the keyword search were in turn studied with respect to their tissue distribution by the "electronic Northern" (eNorthen) method. The eNorthern is based on aligning the sequence of a GOI with an EST (expressed sequence tag) database (Adams et al., *Science* 252:1651, 1991) (http://www.ncbi.nlm.nih.gov/BLAST). The tissue origin of each EST which is found to be homologous to the inserted GOI can be determined and in this way the sum of all ESTs produces a preliminary assessment of the tissue distribution of the GOI. Further studies were carried out only with those GOI which had no homologies to EST from non organ-specific normal tissues. This evaluation also took into account that the public domain contains wrongly annotated cDNA libraries (Scheurle et al., *Cancer Res.* 60:4037-4043, 2000) (www.fau.edu/cmbb/publications/cancergenes6.htm).

The second datamining method utilized was the **cDNA xProfiler** of the NCBI Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/Tissues/xProfiler) (Hillier et al., *Genome Research* 6:807-828, 1996; Pennisi, *Science* 276:1023-1024, 1997). This allows pools of transcriptomes deposited in databases to be related to one another by logical operators. We have defined a pool A to which all expression libraries prepared for example from colon were assigned, excluding mixed libraries. All cDNA libraries prepared from normal tissues other than colon were assigned to pool B. Generally, all cDNA libraries were utilized independently of underlying preparation methods, but only those with a size > 1000 were admitted. Pool B was digitally subtracted from pool A by means of the BUT NOT operator. The set of GOI found in this manner was also subjected to eNorthern studies and validated by a literature research.

This combined datamining includes all of the about 13 000 full-length genes in the public domain and predicts out of these genes having potential organ-specific expression.

All other genes were first evaluated in normal tissues by means of specific RT-PCR. All GOI which had proved to be expressed in non-organ specific normal tissues had to be regarded as false-positives and were excluded from further studies. The remaining ones were studied in a large panel of a wide variety of tumor tissues. The antigens depicted below proved here to be activated in tumor cells.

RNA extraction, preparation of poly-d(T) primed cDNA and conventional RT-PCR analysis

Total RNA was extracted from native tissue material by using guanidium isothiocyanate as chaotropic agent (Chomczynski & Sacchi, *Anal. Biochem.* 162:156-9, 1987). After extraction with acidic phenol and precipitation with isopropanol, said RNA was dissolved in DEPC-treated water.

First strand cDNA synthesis from 2-4 µg of total RNA was carried out in a 20 µl reaction mixture by means of Superscript II (Invitrogen), according to the manufacturer's information. The primer used was a dT(18) oligonucleotide. Integrity and quality of the cDNA were checked by amplification of p53 in a 30 cycle PCR (sense CGTGAGCGCTTCGAGATGTTCCG, antisense CCTAACCAGCTGCCCAACTGTAG, hybridization temperature 67°C).

An archive of first strand cDNA was prepared from a number of normal tissues and tumor entities. For expression studies, 0.5 µl of these cDNAs was amplified in a 30 µl reaction mixture, using GOI-specific primers (see below) and 1 U of HotStarTaq DNA polymerase (Qiagen). Each reaction mixture contained 0.3 mM dNTPs, 0.3 µM of each primer and 3 µl of 10 x reaction buffer. The primers were selected so as to be located in two different exons, and elimination of the interference by contaminating genomic DNA as the reason for false-positive results was confirmed by testing nonreverse-transcribed DNA as template. After 15 minutes at 95°C

to activate the HotStarTaq DNA polymerase, 35 cycles of PCR were carried out (1 min at 94°C, 1 min at the particular hybridization temperature, 2 min at 72°C and final elongation at 72°C for 6 min).
20 µl of this reaction were fractionated and analyzed on an ethidium bromide-stained agarose gel.

The following primers were used for expression analysis of the corresponding antigens at the hybridization temperature indicated.

Claudin18A1 (64°C)
Sense: 5´-GAGGCAGAGTTCAGGCTTCACCGA-3´ (SEQ ID NO: 109)
Antisense: 5´- TGTTGGCTTTGGCAGAGTCC-3´ (SEQ ID NO: 110)
Claudin18A2 (68°C)
Sense1: 5´-GGTTCGTGGTTTCACTGATTGGGATTGC-3´ (SEQ ID NO: 39)
Antisense1: 5´-CGGCTTTGTAGTTGGTTTCTTCTGGTG-3´ (SEQ ID NO: 40)
Sense2: 5´- TGTTTTCAACTACCAGGGGC-3´ (SEQ ID NO: 107)
Antisense2: 5´- TGTTGGCTTTGGCAGAGTCC-3´ (SEQ ID NO: 108)

Preparation of random hexamer-primed cDNA and quantitative real-time PCR

The expression of several genes was quantified by real-time PCR. The PCR products were detected using SYBR Green as intercalating reporter dye. The reporter fluorescence of SYBR Green is suppressed in solution and the dye is active only after binding to double-stranded DNA fragments. The increase in the SYBR Green fluorescence as a result of the specific amplification using GOI-specific primers after each PCR cycle is utilized for quantification. Expression of the target gene is quantified absolutely or relative to the expression of a control gene with constant expression in the tissues to be investigated. Expression was

measured after standardization of the samples against 18s RNA as so-called housekeeping gene using the $\Delta\Delta$-C_t method (PE Biosystems, USA). The reactions were carried out in duplicates and determined in triplicates. The QuantiTect SYBR Green PCR kit (Qiagen, Hilden) was used in accordance with the manufacturer's instructions. The cDNA was synthesized using the high capacity cDNA Archive Kit (PE Biosystems, USA) with use of hexamer primers in accordance with the manufacturer's instructions. Each 5 µl portions of the diluted cDNA were employed in a total volume of 25 µl for the PCR: sense primer 300 nM, antisense primer 300 nM; initial denaturation 95°C for 15 min; 95°C for 30 sec; annealing for 30 sec; 72°C for 30 sec; 40 cycles. The sequences of the primers used are indicated in the respective examples.

Cloning and sequence analysis

Cloning of full-lengths and gene fragments took place by conventional methods. To ascertain the sequence, corresponding antigenes were amplified using the proofreading polymerase pfu (Stratagene). After completion of the PCR, adenosine was ligated by means of HotStarTaq DNA polymerase to the ends of the amplicon in order to clone the fragments in accordance with the manufacturer's instructions into the TOPO-TA vector. The sequencing was carried out by a commercial service. The sequences were analysed using conventional prediction programs and algorithms.

Western blotting

Cells from cell culture (endogenous expression of the target gene or synthesis of the target protein after transfection of an expression vector which encodes the target protein) or tissue samples which might contain the target protein are lysed in a 1% SDS solution. The SDS denatures the proteins present in the lysate. The lysates of an experimental mixture are fractionated according to size by electrophoresis on 8-15%

denaturing polyacrylamide gels (containing 1% SDS) depending on the expected protein size (SDS polyacrylamide gel electrophoresis, SDS-PAGE). The proteins are then transferred by the semi-dry electroblotting method (Biorad) to nitrocellulose membrane (Schleicher & Schüll) on which the desired protein can be detected. For this purpose, the membrane is initially blocked (e.g. with milk powder) and then incubated with the specific antibody in a dilution of 1:20-1:200 (depending on the specificity of the antibody) for 60 minutes. After a washing step, the membrane is incubated with a second antibody coupled to a marker (e.g. enzymes such as peroxidase or alkaline phosphatase) which recognizes the first antibody. After a further washing step, subsequently the target protein is visualized in a color or chemiluminescence reaction on the membrane by means of an enzyme reaction (e.g. ECL, Amersham Bioscience). The result is documented by photographing with a suitable camera.

Analysis of protein modifications usually takes place by Western blotting. Glycosylations, which usually have a size of several kDa, lead to a larger total mass of the target protein, which can be fractionated in the SDS-PAGE. To detect specific O- and N-glycosidic linkages, protein lysates from tissues or cells are incubated before denaturation by SDS with O- or N-glycosidases (in accordance with their respective manufacturer's instructions, e.g. PNgase, endoglycosidase F, endoglycosidase H, Roche Diagnostics). This is followed by Western blotting as described above. Thus, if there is a reduction in the size of a target protein after incubation with a glycosidase it is possible to detect a specific glycosylation and, in this way, also analyse the tumor specificity of a modification. The exact position of the glycosylated amino acid can be predicted with algorithms and prediction programs.

Immunofluorescence

Cells of established cell lines which either synthesize the target protein endogenously (detection of the RNA in RT-PCR or of the protein by Western blotting) or else have been transfected with plasmid DNA before the IF are used. A wide variety of methods (e.g. electroporation, liposome-based transfection, calcium phosphate precipitation) are well established for transfecting cell lines with DNA (e.g. Lemoine et al. Methods Mol. Biol. 1997; 75: 441-7). The transfected plasmid may in the immunofluorescence encode the unmodified protein or else couple various amino acid markers to the target protein. The most important markers are, for example, the fluorescing "green fluorescent protein" (GFP) in its various differentially fluorescing forms and short peptide sequences of 6-12 amino acids for which high-affinity and specific antibodies are available. Cells which synthesize the target protein are fixed with paraformaldehyde, saponin or methanol. The cells can then if required be permeabilized by incubation with detergents (e.g. 0.2% Triton X-100). After the fixation/permeabilization, the cells are incubated with a primary antibody which is directed against the target protein or against one of the coupled markers. After a washing step, the mixture is incubated with a second antibody coupled to a fluorescent marker (e.g. fluorescin, Texas Red, Dako) which binds to the first antibody. The cells labeled in this way are then covered with a layer of glycerol and analysed with the aid of a fluorescence microscope according to the manufacturer's instructions. Specific fluorescence emissions are achieved in this case by specific excitation depending on the substances employed. The analysis normally allows reliable localization of the target protein, the antibody quality and the target protein being confirmed in double stainings to stain in addition to the target protein also the coupled amino acid markers or other marker proteins whose

localization has been described in the literature. GFP and its derivatives represents a special case that can be directly excited and itself fluoresces, so that no antibodies are necessary for the detection.

Immunohistochemistry

IHC serves specifically for (1) being able to estimate the amount of target protein in tumor and normal tissues, (2) analysing how many cells in the tumor and healthy tissue synthesize the target gene, and/or (3) defining the cell type in a tissue (tumor, healthy cells) in which the target protein is detectable.

Different protocols must be used depending on the individual antibody (e.g. "Diagnostic Immunohistochemistry by David J., MD Dabbs ISBN: 0443065667" or in "Microscopy, Immunohistochemistry, and Antigen Retrieval Methods: For Light and Electron Microscopy ISBN: 0306467704").

Immunohistochemistry (IHC) on specific tissue samples serves to detect protein in the corresponding tissue. The aim of this method is to identify the localization of a protein in a functionally intact tissue aggregate. IHC serves specifically for (1) being able to estimate the amount of target protein in tumor and normal tissues, (2) analysing how many cells in tumor and healthy tissue synthesize the target gene, and (3) defining the cell type in a tissue (tumor, healthy cells) in which the target protein is detectable. Alternatively, the amounts of protein of a target gene can be quantified by tissue immunofluorescence using a digital camera and suitable software (e.g. Tillvision, Till-photonics, Germany). The technology has frequently been published, and details of staining and microscopy can therefore be found for example in "Diagnostic Immunohistochemistry" by David J., MD Dabbs ISBN: 0443065667 or "Microscopy, Immunohistochemistry, and Antigen Retrieval Methods: For Light and Electron Microscopy" ISBN: 0306467704. It should be noted that,

because of the properties of antibodies, different protocols have to be used (an example is described below) in order to obtain a valid result.

Ordinarily, histologically defined tumor tissues and, as reference, comparable healthy tissues are employed in the IHC. It is moreover possible to use as positive and negative controls cell lines in which the presence of the target gene is known through RT-PCR analyses. A background control must always be included.

Fixed tissue (e.g. fixation with aldehyde-containing substances, formaldehyde, paraformaldehyde or in alcoholic solutions) or shock-frozen tissue pieces with a thickness of 1-10 µm are applied to a glass support. Paraffin-embedded samples are deparaffinated for example with xylene. The samples are washed with TBS-T and blocked in serum. This is followed by incubation with the first antibody (dilution: 1:2 to 1:2000) for 1-18 hours, with affinity-purified antibodies normally being used. A washing step is followed by incubation with a second antibody which is coupled to an alkaline phosphatase (alternative: for example peroxidase), and is directed against the first antibody, for about 30-60 minutes. This is followed by color reaction using color substrates which are converted by the bound enzymes (cf. for example, Shi et al., *J. Histochem. Cytochem.* 39: 741-748, 1991; Shin et al., *Lab. Invest.* 64: 693-702, 1991). To demonstrate the antibody specificity, the reaction can be blocked by previous addition of the immunogen.

Immunization

(See also Monoclonal Antibodies: A Practical Approach by Philip Shepherd, Christopher Dean ISBN 0-19-963722-9; Antibodies: A Laboratory Manual by Ed Harlow, David Lane ISBN: 0879693142; Using Antibodies: A Laboratory Manual: Portable Protocol NO. by Edward Harlow, David Lane, Ed Harlow ISBN: 0879695447).

The process for preparing antibodies is described briefly below, and details can be found in the cited publications. Firstly, animals (e.g. rabbits) are immunized by a first injection of the desired target protein. The animal's immune response to the immunogen can be enhanced by a second or third immunization within a defined period (about 2-4 weeks after the preceding immunization). Again after various defined periods (first bleeding after 4 weeks, then about every 2 weeks with a total of up to 5 samplings), blood is taken from the animals, and an immune serum is obtained therefrom.

The animals are usually immunized by one of four well-established methods, with other methods also being available. It is moreover possible to immunize with peptides which are specific for the target protein, with the complete protein or with extracellular partial sequences of a protein which can be identified experimentally or via prediction programs.

(1) In the first case, peptides (length: 8-12 amino acids) conjugated to KLH (keyhole limpet hemocyanin) are synthesized by a standardized in vitro method, and these peptides are used for the immunization. Usually, 3 immunizations are carried out with a concentration of 5-1000 µg/immunization. The immunization can also be carried out as service from service providers.

(2) Alternatively, the immunization can be carried out with recombinant proteins. For this purpose, the cloned DNA of the target gene is cloned into an expression vector, and the target protein is synthesized in analogy to the conditions of the particular manufacturer (e.g. Roche Diagnostics, Invitrogen, Clontech, Qiagen) for example cell-free in vitro, in bacteria (e.g. E. coli), in yeast (e.g. S. pombe), in insect cells or in mammalian cells. After

synthesis in one of the systems, the target protein is purified, the purification in this case usually taking place by standardized chromatographic methods. It is also possible in this connection to use for the immunization proteins which have a molecular anchor as aid for purification (e.g. His tag, Qiagen; FLAG tag, Roche Diagnostics; Gst fusion proteins). A large number of protocols is to be found for example in the "Current Protocols in Molecular Biology", John Wiley & Sons Ltd., Wiley Interscience.

(3) If a cell line which synthesizes the desired protein endogenously is available, this cell line can also be used to produce the specific antiserum. In this case, the immunization takes place in 1-3 injections in each case with about 1-5 x 10^7 cells.

(4) The immunization can also take place by injection of DNA (DNA immunization). For this purpose, the target gene is initially cloned into an expression vector so that the target sequence is under the control of a strong eukaryotic promoter (e.g. CMV promoter). Subsequently, 5-100 μg of DNA are transferred as immunogen using a "gene gun" into capillary regions with a strong blood flow in an organism (e.g. mouse, rabbit). The transferred DNA is taken up by the animal's cells, the target gene is expressed, and the animal finally develops an immune response to the target gene (Jung et al., Mol Cells 12:41-49, 2001; Kasinrerk et al., Hybrid Hybridomics 21:287-293, 2002).

Quality control of the polyclonal serum or antibody

Assays based on cell culture with subsequent Western blotting are most suitable for demonstrating specificity (various variations are described for

example in "Current Protocols in Protein Chemistry", John Wiley & Sons Ltd., Wiley InterScience). For the demonstration, cells are transfected with a cDNA, which is under the control of a strong eukaryotic promoter (e.g. cytomegalovirus promoter), for the target protein. A wide variety of methods (e.g. electroporation, liposome-based transfection, calcium phosphate precipitation) are well established for transfecting cell lines with DNA (e.g. Lemoine et al., *Methods Mol. Biol.* 75:441-7, 1997). It is also possible alternatively to use cell lines which express the target gene endogenously (demonstration by target gene-specific RT-PCR). As control, in the ideal case homologous genes are also transfected in the experiment, in order to be able to demonstrate in the following Western blot the specificity of the analysed antibody.

In the subsequent Western blot, cells from cell culture or tissue samples which might contain the target protein are lysed in a 1% SDS solution, and the proteins are denatured thereby. The lysates are fractionated according to size by electrophoresis on 8-15% denaturing polyacrylamide gels (contain 1% SDS) (SDS polyacrylamide gel electrophoresis, SDS-PAGE). The proteins are then transferred by one of a plurality of blotting methods (e.g. semi-dry electroblot; Biorad) to a specific membrane (e.g. nitrocellulose, Schleicher & Schüll). The desired protein can be visualized on this membrane. For this purpose, the membrane is first incubated with the antibody which recognizes the target protein (dilution about 1:20-1:200, depending on the specificity of the antibody) for 60 minutes. After a washing step, the membrane is incubated with a second antibody which is coupled to a marker (e.g. enzymes such as peroxidase or alkaline phosphatase) and which recognizes the first antibody. It is then possible in a color or chemiluminescent reaction to visualize the target protein on the membrane (e.g. ECL, Amersham

Bioscience). An antibody with a high specificity for the target protein should in the ideal case recognize only the desired protein itself.

Various methods are used to confirm the membrane localization of the target protein identified in the in silico approach. An important and well-established method using the antibodies described above is immunofluorescence (IF). Cells of established cell lines which either synthesize the target protein (detection of the RNA in an RT-PCR or of the protein in a Western blot) or else have been transfected with plasmid DNA are used for this. A wide variety of methods (e.g. electroporation, liposome-based transfection, calcium phosphate precipitation) are well established for transfection of cell lines with DNA (e.g. Lemoine et al., *Methods Mol. Biol.* 75:441-7, 1997). The plasmid transfected into the cells can in the immunofluorescence encode the unmodified protein or else couple various amino acid markers to the target protein. The principal markers are, for example, the fluorescent "green fluorescent protein" (GFP) in its various differentially fluorescent forms, short peptide sequences of 6-12 amino acids for which high-affinity and specific antibodies are available, or the short amino acid sequence Cys-Cys-X-X-Cys-Cys which can bind via its cysteines specific fluorescent substances (Invitrogen). Cells which synthesize the target protein are fixed for example with paraformaldehyde or methanol. The cells can then, if required, be permeabilized by incubation with detergents (e.g. 0.2% Triton X-100). The cells are then incubated with a primary antibody which is directed against the target protein or against one of the coupled markers. After a washing step, the mixture is incubated with a second antibody which is coupled to a fluorescent marker (e.g. fluorescin, Texas Red, Dako) and which binds to the first antibody. The cells labeled in this way are then covered with a layer of glycerol and analysed with the

aid of a fluorescence microscope according to the manufacturer's instructions. Specific fluorescence emissions are achieved in this case by specific excitation depending on the substances employed. The analysis usually permits reliable localization of the target protein, the antibody quality and the target protein being confirmed in double stainings to stain in addition to the target protein also the coupled amino acid markers or other marker proteins whose localization has already been described in the literature. GFP and its derivatives represents a special case, being excitable directly and themselves fluorescing. The membrane permeability, which can be controlled through the use of detergents, permits demonstration in the immunofluorescence of whether an immunogenic epitope is located inside or outside the cell. The prediction of the selected proteins can thus be supported experimentally. An alternative possibility is to detect extracellular domains by means of flow cytometry. For this purpose, cells are fixed under non-permeabilizing conditions (e.g. with PBS/Na azide/2% FCS/5 mM EDTA) and analysed in a flow cytometer in accordance with the manufacturer's instructions. Only extracellular epitopes can be recognized by the antibody to be analysed in this method. A difference from immunofluorescence is that it is possible to distinguish between dead and living cells by use of, for example, propidium iodide or Trypan blue, and thus avoid false-positive results.

Affinity purification

Purification of the polyclonal sera took place in the case of the peptide antibodies entirely, or in the case of the antibodies against recombinant proteins in part, as service by the contracted companies. For this purpose, in both cases, the appropriate peptide or recombinant protein was covalently bonded to a matrix, and the latter was, after the coupling, equilibrated with a native buffer (PBS: phosphate buffered saline)

and then incubated with the crude serum. After a further PBS washing step, the antibody was eluted with 100 mM glycine, pH 2.7, and the eluate was immediately neutralized in 2M TRIS, pH 8. The antibodies purified in this way could then be employed for specific detection of the target proteins both by Western blotting and by immunofluorescence.

Preparation of GFP transfectants

For the immunofluorescence microscopy of heterologously expressed tumor-associated antigens, the complete ORF of the antigens was cloned in pGFP-C1 and pGFP-N3 vectors (Clontech). CHO and NIH3T3 cells cultivated on slides were transfected with the appropriate plasmid constructs using Fugene transfection reagent (Roche) in accordance with the manufacturer's instructions and, after 12-24 h, analysed by immunofluorescence microscopy.

Flow-through cytometry

Flow-through cytometric measurements were performed in a manner known *per se* (e.g. Robinson (editor) Handbook of flow cytometry methods. Wiley-Liss, New York, 1993).

Example 1: Identification of claudin-18A1 and claudin-18A2 splice variants as diagnostic and therapeutic cancer targets

The claudin-18 gene codes for a surface membrane molecule having 4 hydrophobic regions. According to prediction programs (TMHMM, TMPred) and in accordance with the topology described for many other members of this family, claudin-18 has four transmembrane domains and two extracellular domains EX1 and EX2, whose extracellular localisation (conformation 1) is shown in Figure 2. The domain D3 which is located between the two extracellular epitopes for claudin-18 and other members of this family is described in the literature as being located intracellularly and this is also predicted by commonly used prediction programs. The N

and C termini are intracellular. Niimi and colleagues (*Mol. Cell. Biol.* 21:7380-90, 2001) described two splice variants of the murine and human claudin-18 which have been described as expressed selectively in lung tissue (claudin-18A1) and in stomach tissue (claudin-18A2), respectively. These variants differ in the N terminus.

It was investigated according to the invention how far the splice variants claudin-18A2 (SEQ ID NO:7) and claudin-18A1 (SEQ ID NO:117), and their respective translation products (SEQ ID NO:16 and 118), can be used as markers or therapeutic target structures for tumors. A quantitative PCR able to distinguish between the two variants was established by selecting A1-specific (SEQ ID NO:109, 110) and A2-specific (SEQ ID NO:107, 108) primer pairs. The A2 splice variant was additionally tested with a second primer pair in a conventional PCR (SEQ ID NO:39, 40). The A1 variant is described to be active only in healthy lung tissue. However, it was surprisingly found according to the invention that the A1 variant is also active in the gastric mucosa (fig. 3). Stomach and lung are the only normal tissues showing significant activation. All other normal tissues are negative for claudin-A1. On investigating tumors, it was surprisingly found that claudin-A1 is highly activated in a large number of tumor tissues. Particularly strong expression is to be found in stomach tumors, lung tumors, pancreatic tumors, esophageal tumors (fig. 3), ENT tumors and prostate tumors. The claudin-A1 expression levels in ENT, prostate, pancreatic and esophageal tumors are 100-10 000 higher than the levels in the corresponding normal tissues. The oligonucleotides used to investigate the claudin-A2 splice variant specifically enable this transcript to be amplified (SEQ ID NO:39, 40 and 107, 108). Investigation revealed that the A2 splice variant is expressed in none of the more than 20 normal tissues investigated apart from gastric mucosa and to a small extent also testis tissue (fig. 4). We

have found that the A2 variant is also, like the A1 variant, activated in many tumors (fig. 4). These include stomach tumors, pancreatic tumors, esophageal tumors and liver tumors. Although no activation of claudin-18A2 is detectable in healthy lung, it was surprisingly found that some lung tumors express the A2 splice variant.

Table 1A. Expression of claudin-18A2 in normal and tumor tissues

Normal tissue	Expression
Brain	-
Cerebellum	-
Myocardium	-
Skeletal muscle	-
Endometrium	-
Stomach	+++
Colon	-
Pancreas	-
Kidney	-
Liver	-
Testis	+
Thymus	-
Breast	-
Ovary	-
Uterus	-
Skin	-
Lung	-
Thyroid	-
Lymph nodes	-
Spleen	-
PBMC	-
Esophagus	-

Tumor type	Expression
Colon	-
Pancreas	++
Esophagus	++
Stomach	+++
Lung	++
Breast	-
Ovary	-
Endometrium	n.i.
ENT	++
Kidney	-
Prostate	-

Table 1B. Expression of claudin-18A1 in normal and tumor tissues

Normal tissue	**Expression**
Brain	-
Cerebellum	-
Myocardium	-
Muscle	-
Endometrium	-
Stomach	+++
Colon	-
Pancreas	-
Kidney	-
Liver	-
Testis	+
Thymus	-
Breast	-
Ovary	-
Uterus	-
Skin	-
Lung	+++
Thyroid	-
Lymph nodes	-
Spleen	-
PBMC	-
Esophagus	-

Tumor type	**Expression**
Colon	-
Pancreas	++
Esophagus	++
Stomach	+++
Lung	++
Breast	+
Ovary	n.i.
Endometrium	n.i.
ENT	++
Kidney	-
Prostate	++

Conventional PCR as independent control investigation also confirmed the results of the quantitative PCR. The oligonucleotides (SEQ ID NO:39, 40) used for this permit specific amplification of the A2 splice variant. It was shown according to the invention that most gastric tumors and half of the tested pancreatic tumors showed strong expression of this splice variant (fig. 1). By contrast, expression is not detectable in other tissues by conventional PCR. In particular, there is no expression in important normal tissues such as lung, liver, blood, lymph nodes, breast and kidney (tab. 1).

The splice variants thus represent according to the invention highly specific molecular markers for tumors of the upper gastrointestinal tract as well as lung tumors, ENT tumors, prostate tumors and metastases thereof. These molecular markers can be used according to the invention for detecting tumor cells. Detection of the tumors is possible according to the invention with the oligonucleotides described (SEQ ID NO:39, 40, 107-110). Particularly suitable oligonucleotides are primer pairs of which at least one binds under stringent conditions to a segment of the transcript which is 180 base pairs long and is specific for one (SEQ ID NO:8) or the other splice variant (SEQ ID NO:119).

These genetic products are attractive therapeutic target structures since due to the fact that they are missing in most toxicity relevant organs no side effects on these organs are to be expected, while due to the strong activation in cells of the cancer types mentioned strong binding to these cells and mediation of corresponding cell damaging effects can be expected.

In order to confirm these data at the protein level, claudin-specific antibodies and immune sera were generated by immunizing animals. The N-terminal

extracellular domain EX1 differs in sequence in the two splice variants A1 and A2 (SEQ ID NO:111 for A1 and SEQ ID NO:112 for A2). The C-terminal extracellular domain EX2 is identical for both variants (SEQ ID NO:137). To date, no antibodies which bind to the extracellular domains of claudin-18 have yet been described. Also no antibodies which are able to discriminate specifically between A1 and A2 variants have yet been described. According to the invention, peptide epitopes and protein fragments which are located extracellularly and are specific for variant A1 or A2 or occur in both variants were selected for the immunization in order to produce antibodies. The following peptides, inter alia, were selected for the immunization in order to produce antibodies:

SEQ ID NO:17: DQWSTQDLYN (N-terminal extracellular domain, A2-specific, binding independent of glycosylation)

SEQ ID NO:18: NNPVTAVFNYQ (N-terminal extracellular domain, A2-specific, binding mainly to unglycosylated form, N37)

SEQ ID NO:113: STQDLYNNPVTAVF (N-terminal extracellular domain, A2-specific, binding only to non-glycosylated form, N37)

SEQ ID NO:114: DMWSTQDLYDNP (N-terminal extracellular domain, A1-specific)

SEQ ID NO:115: CRPYFTILGLPA (N-terminal extracellular domain, mainly specific for A1)

SEQ ID NO:116: TNFWMSTANMYTG (C-terminal extracellular domain, recognizes both A1 and A2).

Inter alia, antibodies could be produced which selectively recognize the N terminal domain of the splice variant claudin-18A1 but not the A2 variant (fig. 8). Using epitopes for immunizations located in the C terminal extracellular domain which is identical in both splice variants, antibodies could be produced which recognize both variants (fig. 7).

The data for a A2-specific antibody produced by immunization with SEQ ID NO:17 are shown by way of example. The specific antibody can be utilized under various fixation conditions for immunofluorescence investigations. With comparative stainings of RT-PCR-positive and negative cell lines, in an amount which is readily detectable, the corresponding protein can be specifically detected inter alia in the gastric tumor, esophageal tumor and pancreatic tumor cell lines typed as positive (fig. 5). The endogenous protein is membrane-located and forms relatively large focal aggregates on the membrane (fig. 5). This antibody was used for immunohistochemical stainings of human tissues. The selective tissue distribution of this protein was confirmed. A large series of different normal tissues was investigated in most of which claudin-18A2 protein was not detectable as shown by way of example for liver, lung, kidney and colon. Activation of this protein was only found in normal stomach tissue (fig. 12). Surprisingly, the A2 variant of claudin-18 was detectable in the differentiated cells of stomach mucosa but not in stem cells. Differentiated stomach mucosa cells are subject to permanent regeneration. Physiologically, the total stomach epithelium is continuously replaced from the stem cells of the stomach. This supports the usefulness of the A2 variant as therapeutic target structure since it was shown according to the invention that stem cells of the stomach as the indispensable cell population of stomach mucosa do not harbour the A2 variant as all other healthy organs and, thus, are not attacked by a substance which is specifically directed against the A2 variant. Using this antibody, the A2 variant of claudin-18 was detected in a series of human tumors (fig. 13), in particular in tumors of stomach, esophagus and lung, which attracted already attention in RT-PCR investigations. According to the invention, these tumors are therapeutically accessible. The antibody described above was additionally employed for

protein detection in Western blotting. As expected, protein is detected only in stomach and in no other normal tissue, not even lung where only the A1 variant is activated (fig. 9). The comparative staining of stomach tumors and adjacent normal stomach tissue from patients surprisingly revealed that claudin-18 A2 has a smaller mass weight in all stomach tumors in which this protein is detected (fig. 10, left). It was found according to the invention in a series of experiments that a band also appears at this position when lysate of normal stomach tissue is treated with the deglycosylating agent PNGase F (fig. 10, right). Whereas exclusively the glycosylated form of the A2 variant is detectable in all normal stomach tissues, A2 is detectable as such in more than 60% of the investigated gastric tumors, in particular exclusively in the deglycosylated form. Although the A2 variant of claudin-18 is not detected in normal lung even at the protein level, it is to be found in bronchial tumors, as also previously in the quantitative RT-PCR. Once again, only the deglycosylated variant is present (fig. 11).

Claudin-18 is a highly selective differentiation antigen of stomach (variant A2) or lung and stomach (variant A1). Our data indicate that it is obviously subject to tumor-associated alterations of the glycosylation machinery and that in tumors a specific form of the variant A2 is produced which is deglycosylated. The results of the PNGaseF-treatment show that claudin-18A2 differs in its N glycosylation in tumor and normal tissue.

The glycosylation of an epitope can prevent binding of an antibody specific for this epitope and can in the present case contribute to the inability of such an antibody to bind to claudin-18A2 in normal tissues but to the exclusive binding to the non-glycosylated form in cancer cells. To produce antibodies according to the

invention which selectively bind to non-glycosylated epitopes, this was considered in selecting the immunogens. According to the invention, different regions of claudin-18A2 were identified which can be present in tumor and normal tissue in a differentially glycosylated form. Among others, the regions comprising the amino acids 37, 38, 45, 116, 141, 146, 205 of claudin-18A2 were identified as potential glycosylation sites for claudin-18A2 (fig. 2, below). According to the invention, tumor cells and normal tissues differ in glycosylation at one or more of these positions. Most of these regions do not represent a classical glycosylation site but contain asparagin, serine and threonine which infrequently can also be glycosylated (prediction of fig. 2, below). Both variants of claudin-18 have a unique classical glycosylation motive in the D3 domain which according to the literature and commonly used prediction algorithms is supposed to be intracellularly located.

However, for PMP 22 which is a tetraspanine which is structurally similar to claudin-18, it was shown that the hydrophobic membrane domains 2 and 3 do not span entirely through the cell membrane but intercalate only partially in the plasma membrane (Taylor et al., J. Neurosc. Res. 62:15-27, 2000). For this reason, the entire region between the two outer transmembrane domains of PMP22 is located extracellularly. The possibility for such a topology was hypothesized and verified for claudin-18A2. To this end, three constructs were prepared which each carried a marker sequence (His or HA tag) in one of the EX1, EX2 or D3 domains (fig. 15, top). These were transfected into cell lines and it was tested whether an antibody directed against these marker sequences binds to non-permeabilized cells which requires that the corresponding region of the protein is located topologically in an extracellular manner. Since all three regions of the molecule were determined to be

extracellular by flow-through cytometry (fig. 15, below), it was confirmed that claudin-18A2 can be present in a conformation having two transmembrane domains and one large extracellularly located domain (fig. 2, conformation 2). This conformation is biochemically and therapeutically relevant since it contains additional binding sites for therapeutic antibodies (SEQ ID NO: 142, 143).

According to the invention, antibodies are preferably produced which discriminate between glycosylated and non-glycosylated variants of claudin-18A2. These have a particularly high specificity for tumor cells. In preparing antibodies which are specific for the glycosylation also these different conformations besides the glycosylation domains were considered.

Preferably, protein fragments from the D3 region of claudin-18A2 are suitable for immunizing animals in a non-limiting manner. This is shown for two antibodies mAB1 and mAB2 by way of example (fig. 17). The binding properties of these antibodies to cell lines which express the A1 or A2 variant of claudin-18 were investigated. It was shown that claudin-18A2 is accessible for antibodies on the cell surface. According to the invention, such antibodies are specific for the A2 variant and do not bind to the A1 variant (fig. 17). Short foreign sequences (myc tag) were each introduced into the region of the extracellular domains Ex1 and Ex2 (fig. 43). For example, it is shown for mAB1 that the binding properties of the antibody are not affected thereby and that the actual epitope is located in the D3 domain.

The antibodies generated can be utilized diagnostically as well as therapeutically. Immune sera such as the one described herein (directed against peptide SEQ ID NO: 17) can be utilized diagnostically, for example, for Western blotting. According to the invention,

antibodies which do not bind to the glycosylated epitope can be produced by immunizing with peptides which contain at least one of these regions (for example, peptide SEQ ID NO: 113 (fig. 6), peptide SEQ ID NO: 142-145). According to the invention, such antibodies specifically bind to the deglycosylated epitopes on tumor cells. The glycosylation which is missing compared to normal tissues at one of the positions mentioned might also be due to a secondary endogenous deglycosylation in tumor cells. Such a deglycosylation is associated with a Asn (N) → Asp (D) transformation of the respective amino acid. For the production of antibodies against tumor-associated variants which are modified in such a manner, peptides derived from claudin-18A2 can thus be used according to the invention in which the amino acid Asn (N) at at least one of the positions 37, 38, 45, 116, 141, 146, 205 of the claudin-18A2 peptide is substituted by Asp (D) (e.g. SEQ ID NO: 146-150). It is possible in particular to employ such antibodies therapeutically because they are highly selective for tumor cells. The produced antibodies can be used directly also for producing chimeric or humanized recombinant antibodies. This can also take place directly with antibodies obtained from rabbits (concerning this, see J Biol Chem. 2000 May 5;275(18):13668-76 by Rader C, Ritter G, Nathan S, Elia M, Gout I, Jungbluth AA, Cohen LS, Welt S, Old LJ, Barbas CF 3rd. "The rabbit antibody repertoire as a novel source for the generation of therapeutic human antibodies"). For this purpose, lymphocytes from the immunized animals were preserved. The amino acids 1-47 (SEQ ID NO:19 and 120) also represent particularly good epitopes for immunotherapeutic methods such as vaccines and the adoptive transfer of antigen-specific T lymphocytes.

Patentkrav

1. Anvendelse af en ethylgruppe i en fremgangsmåde til detektering af tumorceller, der udtrykker et tumorassocieret antigen, hvor antistoffet binder til det tumorassocierede antigen, hvor antistoffet kan opnås ved immunisering med et peptid eller polypeptid med en aminosyresekvens, der er udvalgt fra gruppen bestående af SEQ ID NO: 142-148, og hvor det tumorassocierede antigen udtrykt i tumorvæv har aminosyresekvensen ifølge SEQ ID NO: 16 og sammenlignet med det raske normale væv er en deglycosyleret variant af det tumorassocierede antigen, hvor glycosyleringen er en N-glycosylering og tumoren er en mavetumor eller en lungetumor, og fremgangsgangen omfatter detektering af den deglycosylerede variant af det tumorassocierede antigen i en biologisk prøve.

2. Anvendelse ifølge krav 1, hvor antistoffet er et monoklonalt antistof.

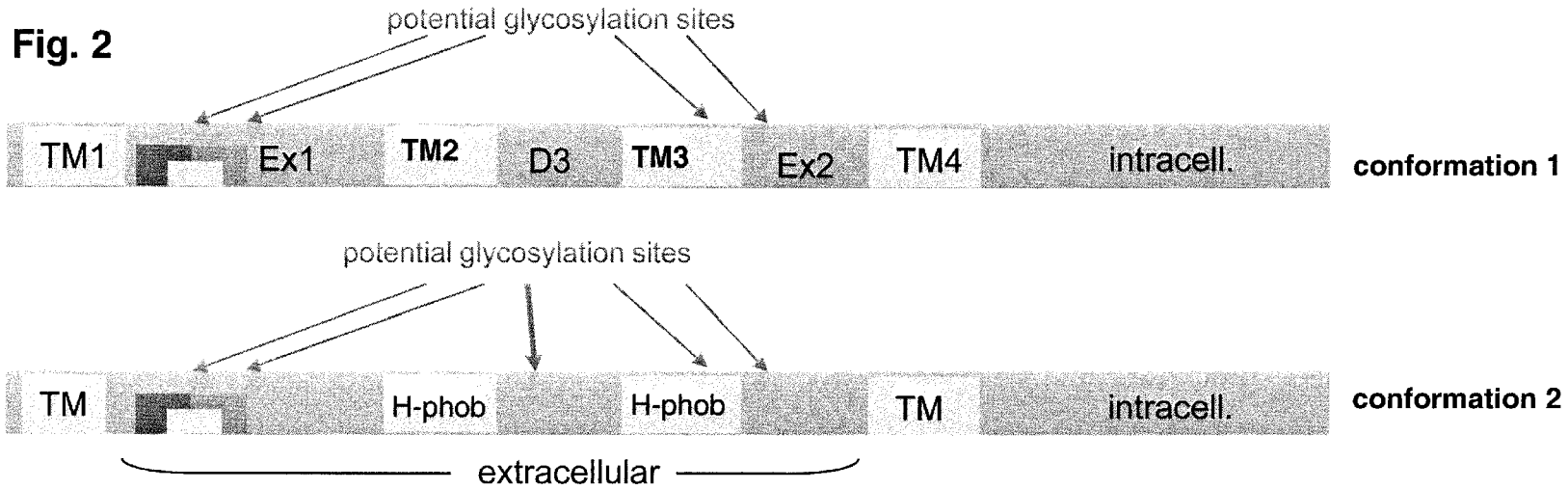
3. Anvendelse ifølge krav 2, hvor antistoffet er et kimærisk eller humaniseret antistof eller et fragment af et antistof, der selektivt binder til det tumorassocierede antigen.

4. Anvendelse ifølge et hvilket som helst af kravene 1 til 3, hvor antistoffet er koblet til et diagnostisk middel.

Fig. 1


M
gastric tumors
pancreatic tumors
ovarian tumors
M
stomach
esophagus
ovary
neg. control

Fig. 2



predicted glycosylation sites (amino acid positions)

Claudin18A2

SeqName	Position	Ranking of the glycosylation sites	Result
Sequence	37	0.7219 (9/9)	++
Sequence	38	0.6502 (8/9)	+
Sequence	45	0.6026 (8/9)	+
Sequence	116	0.5713 (7/9)	+
Sequence	141	0.6348 (7/9)	+
Sequence	146	0.5187 (6/9)	+
Sequence	153	0.4696 (5/9)	-
Sequence	205	0.6011 (8/9)	+
Sequence	234	0.3960 (8/9)	-
Sequence	237	0.4602 (6/9)	-

Fig. 3

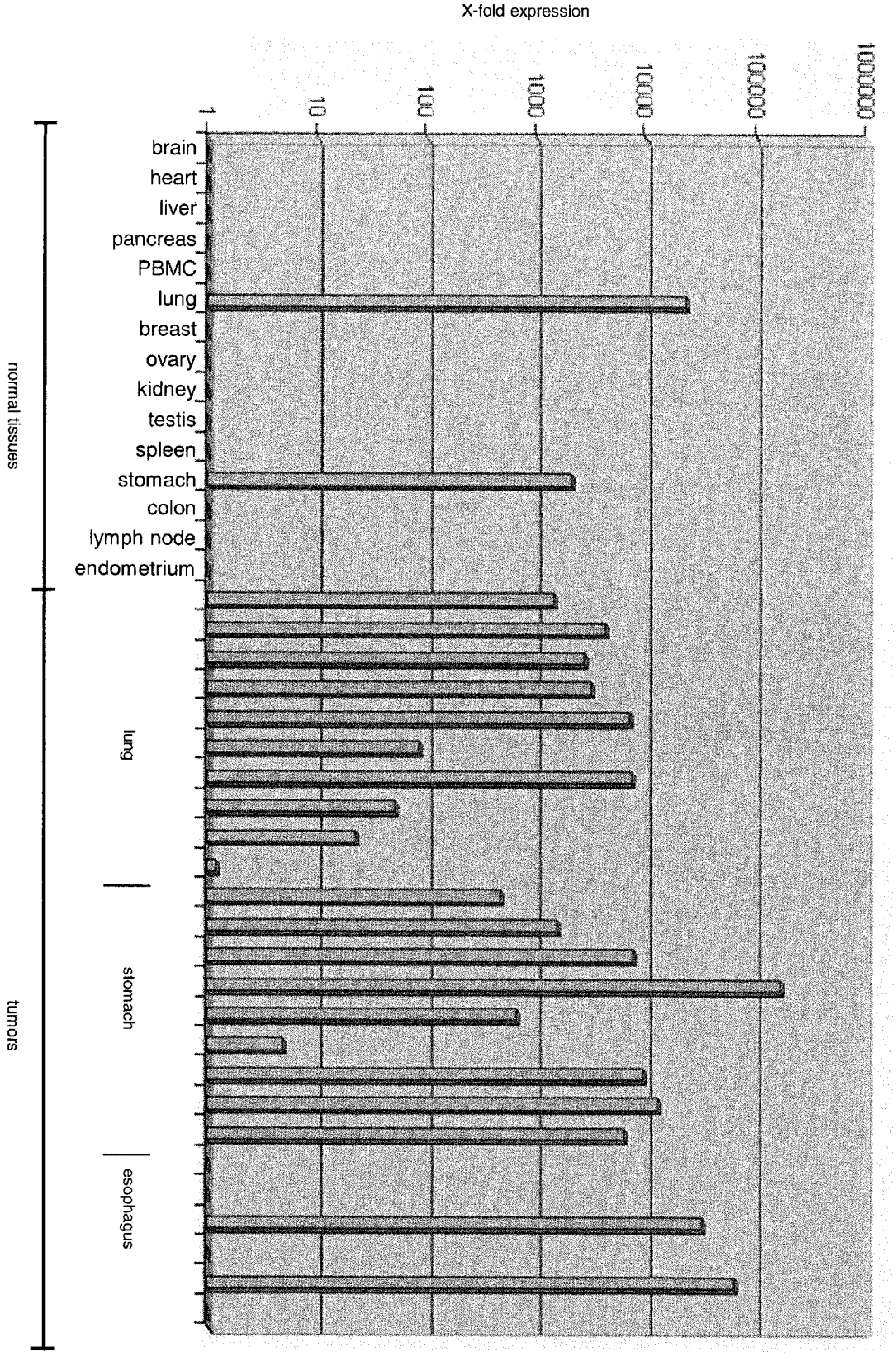
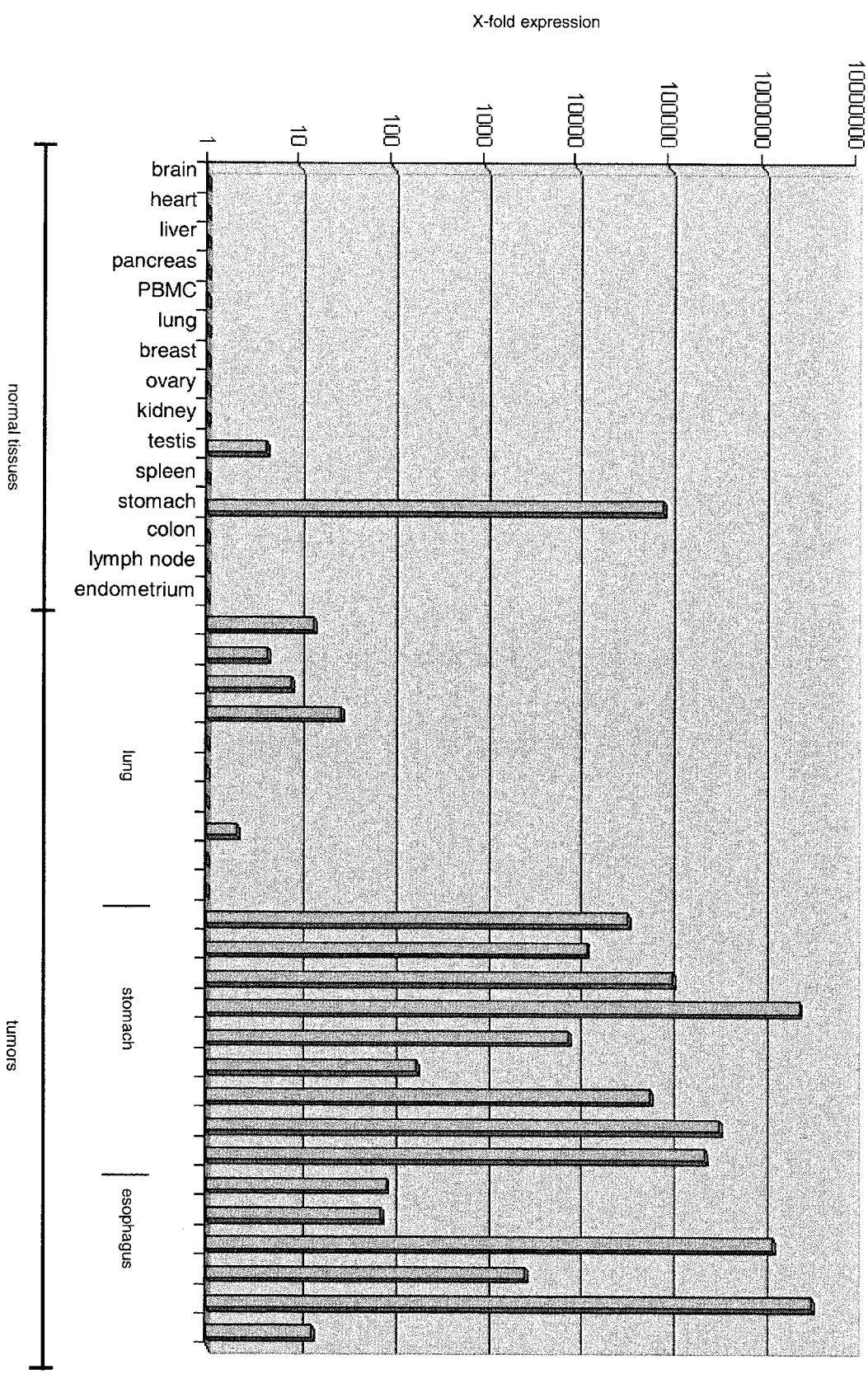

X-fold expression
1
10
100
1000
10000
100000
1000000
brain
heart
liver
pancreas
PBMC
lung
breast
ovary
kidney
testis
spleen
stomach
colon
lymph node
endometrium
normal tissues
lung
stomach
esophagus
tumors

Fig. 4


X-fold expression
1
10
100
1000
10000
100000
1000000
10000000
brain
heart
liver
pancreas
PBMC
lung
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testis
spleen
stomach
colon
lymph node
endometrium
normal tissues
lung
stomach
esophagus
tumors

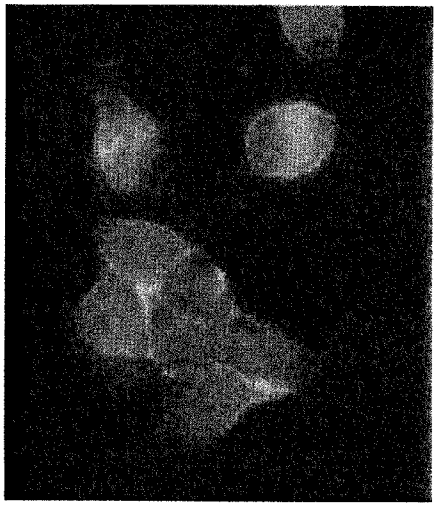


Fig. 5A

Fig. 5B

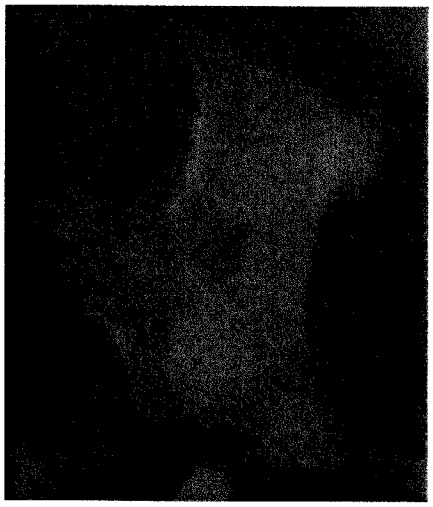


Fig. 5C

Fig. 5D

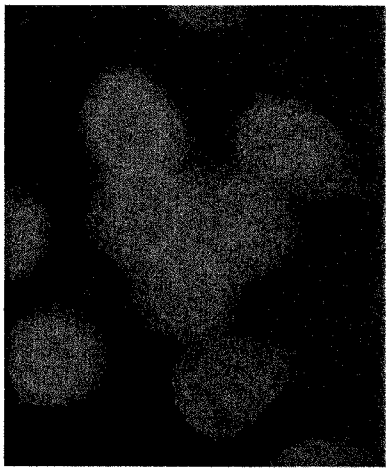


Fig. 6A

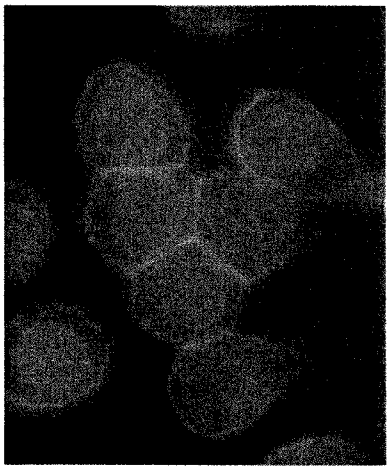


Fig. 6B

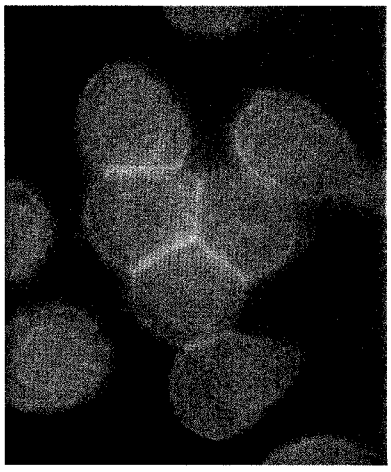
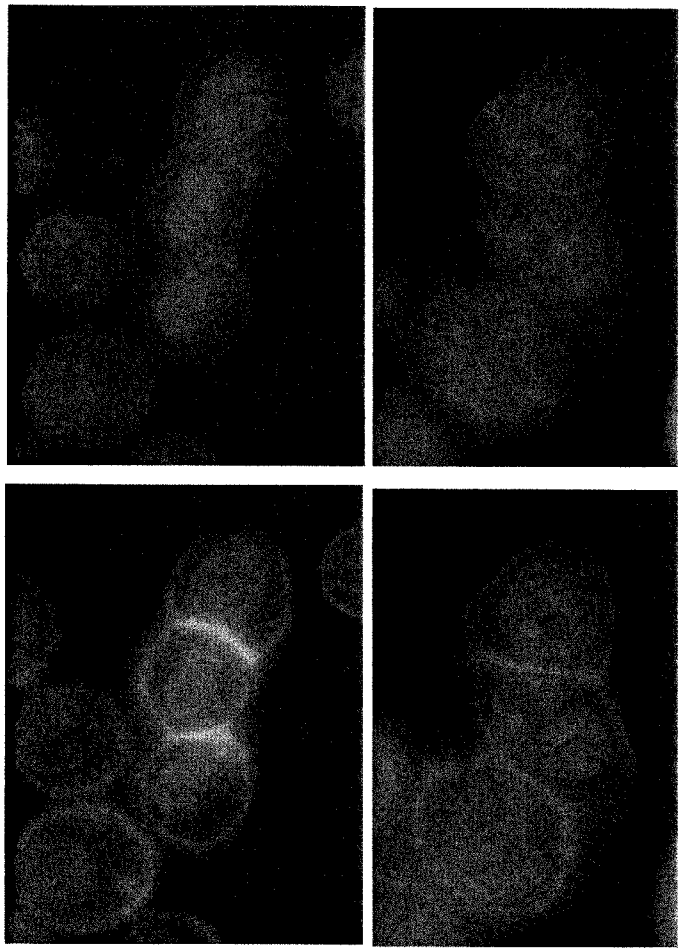


Fig. 6C

Fig. 7



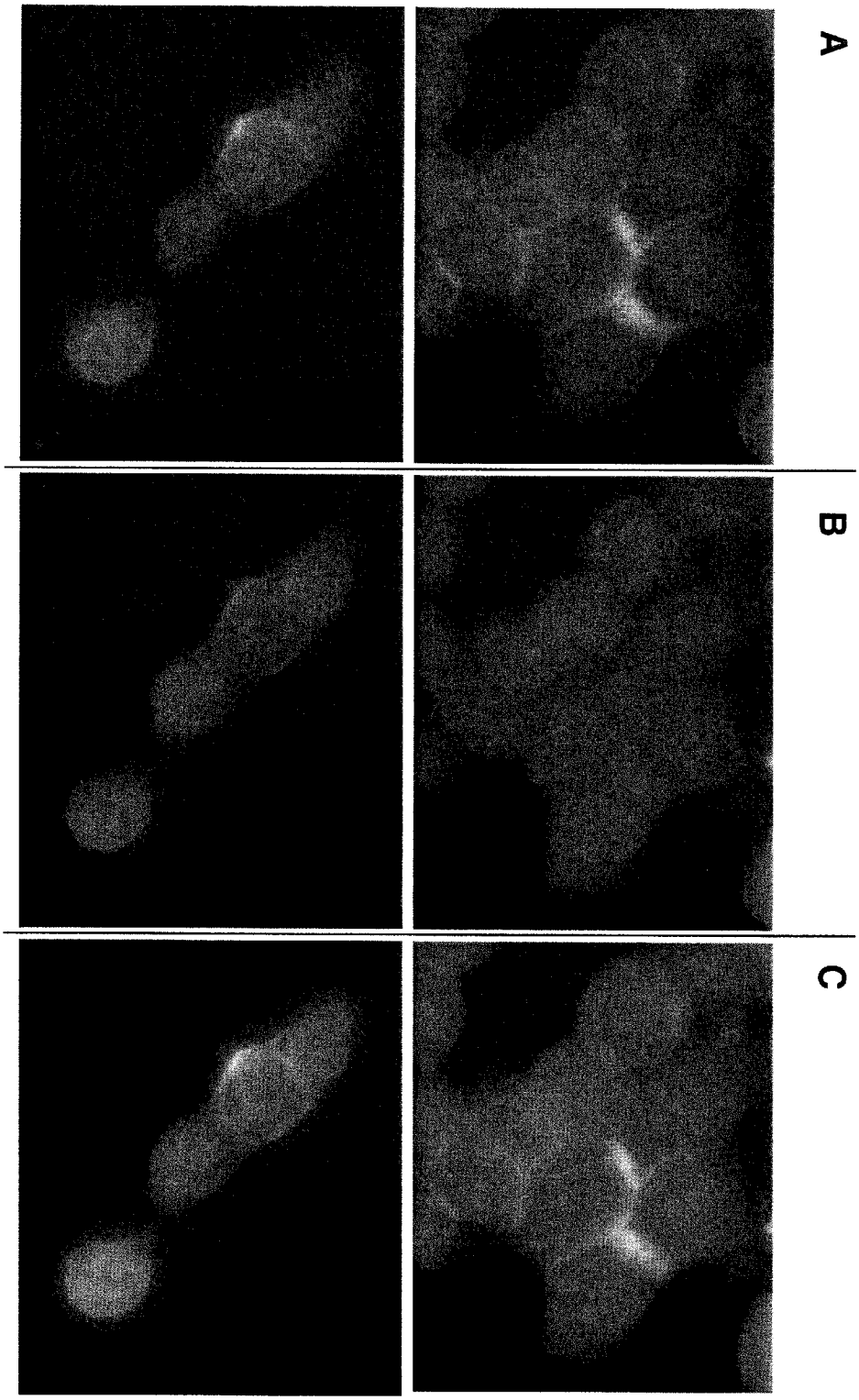


Fig. 8

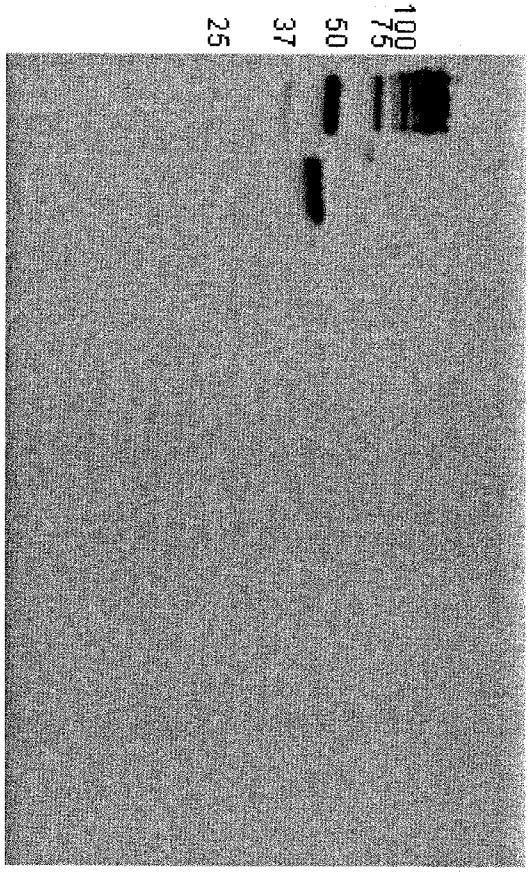


Fig. 9

Fig. 10A

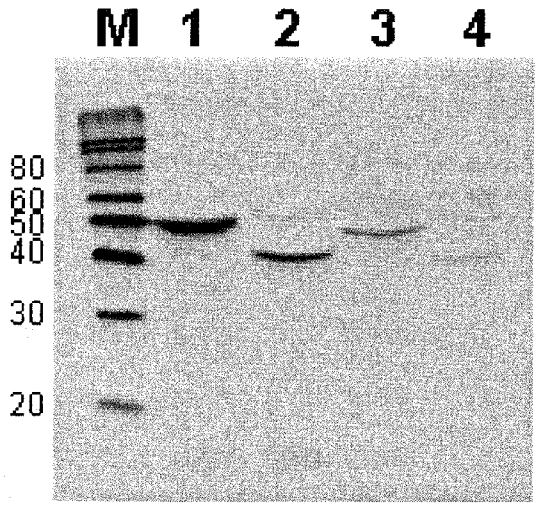


Fig. 10B

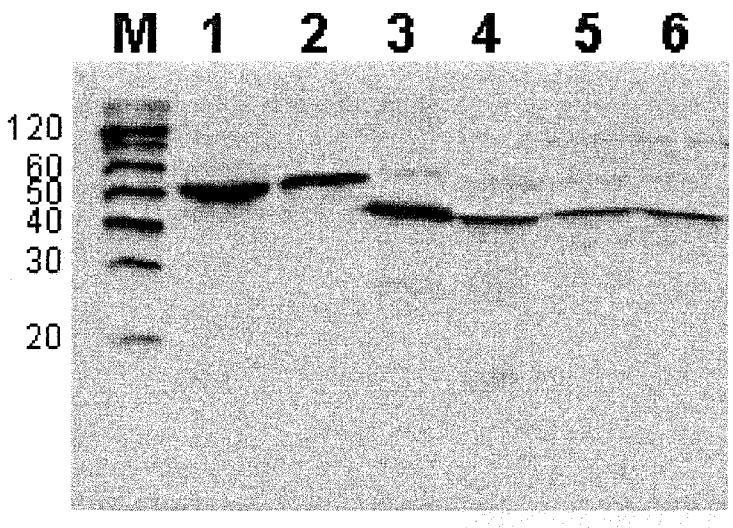


Fig. 10C

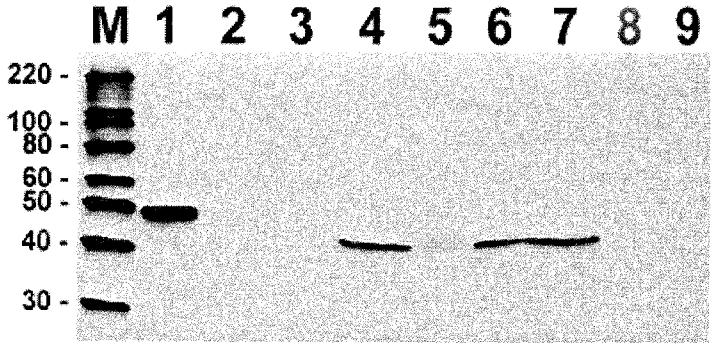


Fig. 10D

tissue specificity of the cell lines	positive / tested cell lines
stomach	4 / 5
esophagus	1 / 3
lung	2 / 5
pancreas	4 / 5
breast	1/ 4
colon	1 / 3
kidney	0 / 1
skin	0 / 1
ovary	1 / 2
liver	0 / 1

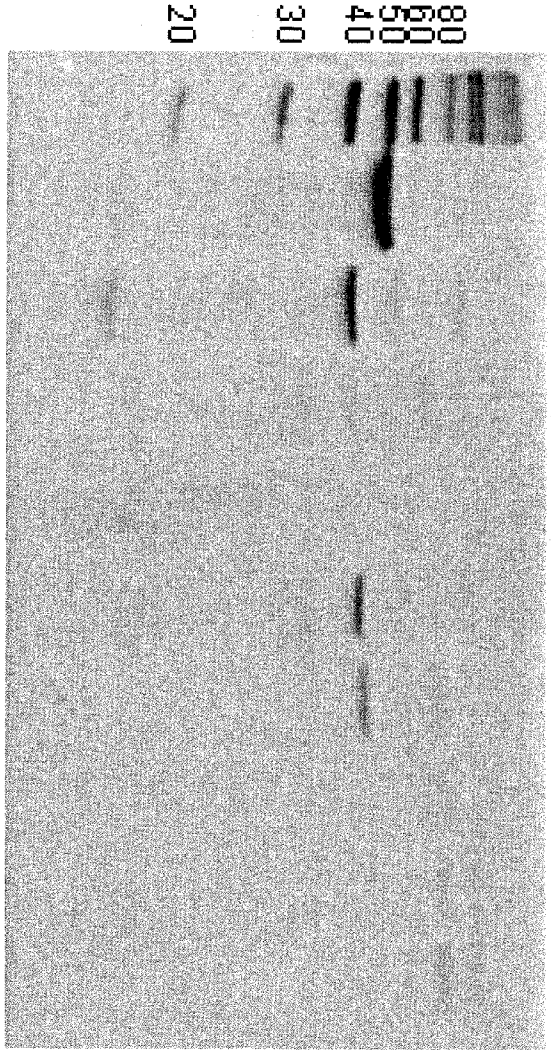


Fig. 11

Fig. 12

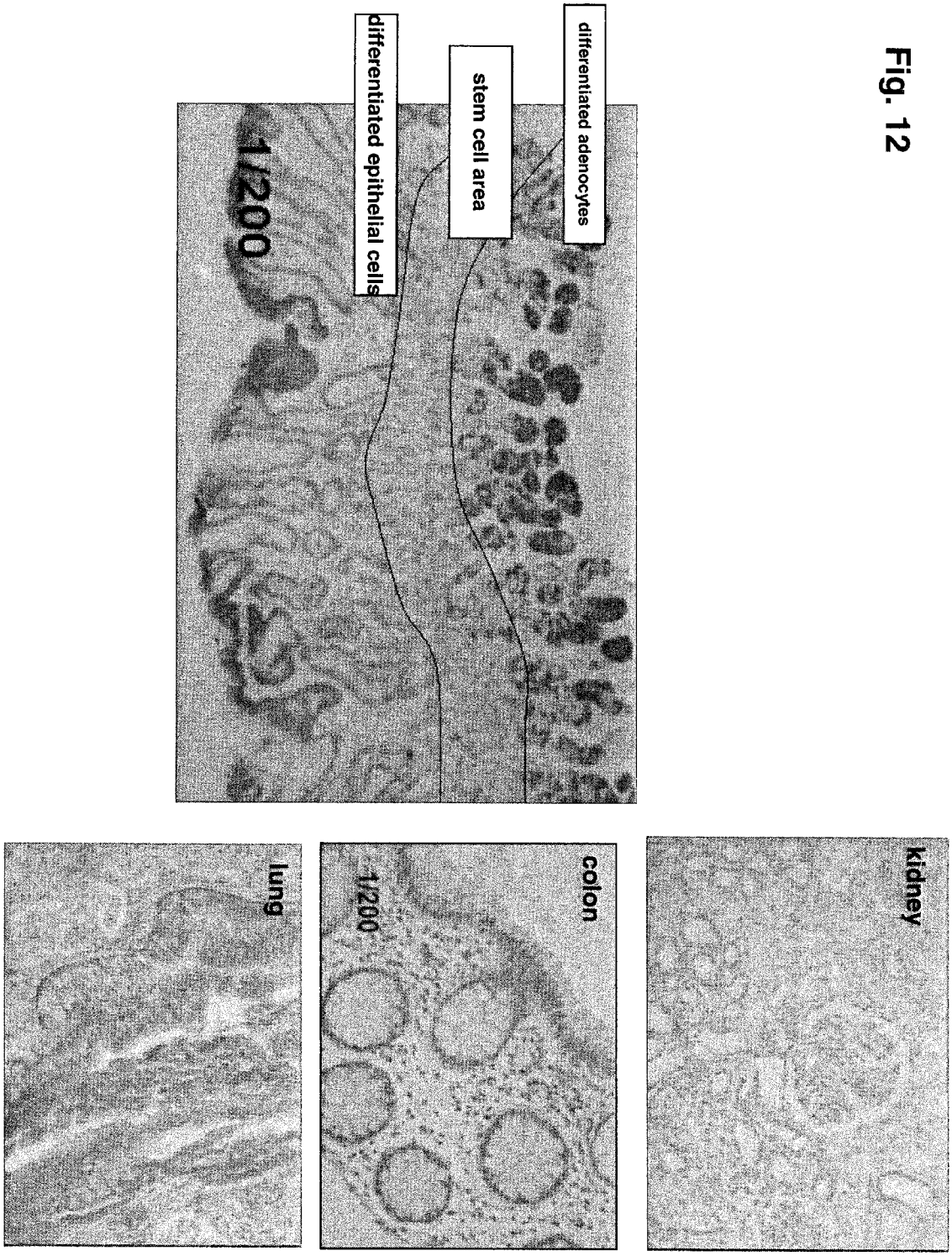

differentiated adenocytes
stem cell area
differentiated epithelial cells
1/200
kidney
colon
1/200
lung

Fig. 13C

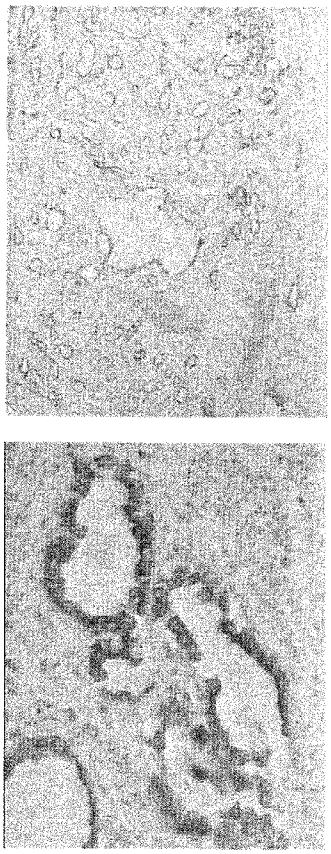


Fig. 13B

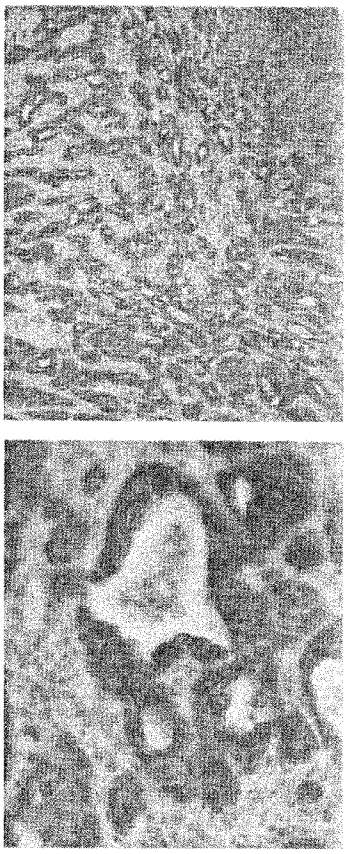


Fig. 13A

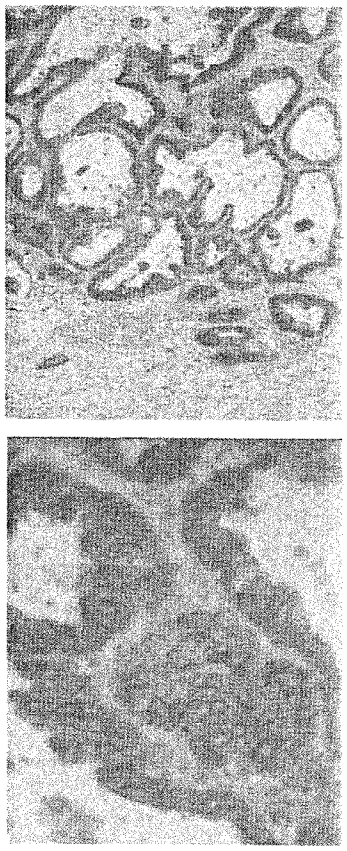


Fig. 13D

tissue	tumor	
	tumor type	positive/ total
stomach	AdenoCa	9/10
lung	AdenoCa	11/12
lung	SCC	0/2
esophagus	AdenoCa	5/5
esophagus	SCC	0/3
prostate		1/3
breast		1/3
kidney	RCC	0/3
colon		0/3
brain	glioblastoma	0/3

Fig. 14

ID	Sequence
#7	ATGGCCGTGACTGCCTGTCAGGGCTTGGGGTTCGTGGTTTCACTGATTGGGATTGCGGGCATCATTGCTG CCACCTGCATGGACCAGTGGAGCACCCAAGACTTGTACAACAACCCCGTAACAGCTGTTTTCAACTACCA GGGGCTGTGGCGCTCCTGTGTCCGAGAGAGCTCTGGCTTCACCGAGTGCCGGGGCTACTTCACCCTGCTG GGGCTGCCAGCCATGCTGCAGGCAGTGCGAGCCCTGATGATCGTAGGCATCGTCCTGGGTGCCATTGGCC TCCTGGTATCCATCTTTGCCCTGAAATGCATCCGCATTGGCAGCATGGAGGACTCTGCCAAAGCCAACAT GACACTGACCTCCGGGATCATGTTCATTGTCTCAGGTCTTTGTGCAATTGCTGGAGTGTCTGTGTTTGCC AACATGCTGGTGACTAACTTCTGGATGTCCACAGCTAACATGTACACCGGCATGGGTGGGATGGTGCAGA CTGTTCAGACCAGGTACACATTTGGTGCGGCTCTGTTCGTGGGCTGGGTCGCTGGAGGCCTCACACTAAT TGGGGGTGTGATGATGTGCATCGCCTGCCGGGGCCTGGCACCAGAAGAAACCAACTACAAAGCCGTTTCT TATCATGCCTCAGGCCACAGTGTTGCCTACAAGCCTGGAGGCTTCAAGGCCAGCACTGGCTTTGGGTCCA ACACCAAAAACAAGAAGATATACGATGGAGGTGCCCGCACAGAGGACGAGGTACAATCTTATCCTTCCAA GCACGACTATGTGTAA
#8	tgcgccaccatggccgtgactgcctgtcagggcttggggttcgtggtttcactgattggg attgcgggcatcattgctgccacctgcatggaccagtggagcacccaagacttgtacaac aaccccgtaacagctgttttcaactaccaggggctgtggcgctcctgtgtccgagagagc
#16	MAVTACQGLGFVVSLIGIAGIIAATCMDQWSTQDLYNNPVTAVFNYQGLWRSCVRESSGFTECRGYFTLL GLPAMLQAVRALMIVGIVLGAIGLLVSIFALKCIRIGSMEDSAKANMTLTSGIMFIVSGLCAIAGVSVFA NMLVTNFWMSTANMYTGMGGMVQTVQTRYTFGAALFVGWVAGGLTLIGGVMMCIACRGLAPEETNYKAVS YHASGHSVAYKPGGFKASTGFGSNTKNKKIYDGGARTEDEVQSYPSKHDYV*
#17	DQWSTQDLYN
#18	NNPVTAVFNYQ
#19	MAVTACQGLGFVVSLIGIAGIIAATCMDQWSTQDLYNNPVTAVFNYQ
#39	GGTTCGTGGTTTCACTGATTGGGATTGC
#40	CGGCTTTGTAGTTGGTTTCTTCTGGTG
#107	TGTTTTCAACTACCAGGGGC
#108	TGTTGGCTTTGGCAGAGTCC
#109	GAGGCAGAGTTCAGGCTTCACCGA
#110	TGTTGGCTTTGGCAGAGTCC
#111	TGMDMWSTQDLYDNPVTSVFQYEGLWRSCVRQSSGFTECRPYFTILGLPAMLQAVR
#112	DQWSTQDLYNNPVTAVFNYQGLWRSCVRESSGFTECRGYFTLL GLPAMLQAVR
#113	STQDLYNNPVTAVF
#114	DMWSTQDLYDNP
#115	CRPYFTILGLPA
#116	TNFWMSTANMYTG
#117	gccaggatca tgtccaccac cacatgccaa gtggtggcgt tcctcctgtc catcctgggg ctggccggct gcatcgcggc caccgggatg gacatgtgga gcacccagga cctgtacgac aaccccgtca cctccgtgtt ccagtacgaa gggctctgga ggagctgcgt gaggcagagt tcaggcttca ccgaatgcag gccctatttc accatcctgg gacttccagc catgctgcag gcagtgcgag ccctgatgat cgtaggcatc gtcctgggtg ccattggcct cctggtatcc atctttgccc tgaaatgcat ccgcattggc agcatggagg actctgccaa agccaacatg acactgacct ccgggatcat gttcattgtc tcaggtcttt gtgcaattgc tggagtgtct gtgtttgcca acatgctggt gactaacttc tggatgtcca cagctaacat gtacaccggc atgggtggga tggtgcagac tgttcagacc aggtacacat ttggtgcggc tctgttcgtg ggctgggtcg ctggaggcct cacactaatt gggggtgtga tgatgtgcat cgcctgccgg ggcctggcac cagaagaaac caactacaaa gccgtttctt atcatgcctc aggccacagt gttgcctaca agcctggagg cttcaaggcc agcactggct ttgggtccaa caccaaaaac aagaagatat acgatggagg tgcccgcaca gaggacgagg tacaatctta tccttccaag cacgactatg tgtaatgctc taagacctct cagcac
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	tcaggcttca ccgaatgcag gccctatttc accatcctgg gacttcc
#120	MSTTTCQVVAFLLSILGLAGCIAATGMDMWSTQDLYDNPVTSVFQYEGLWRSCVRQSSGFTECRPYFTI
#137	NMLVTNFWMSTANMYTGMGGMVQTVQTRYTFG
#142	almivgivlgaigllvsifalkcirigsmedsakanmtltsgimfivsglcaiagvsvfanmlvtnfwmst anmytgmggmvqtvqtrytfgaalfvgwvaggltliggvmmciac
#143	rigsmedsakanmtltsgimfivs
#144	Akanmtlt
#145	Medsakanmtltsg
#146	Medsakadmtltsg
#147	sakadmtlt
#148	akadmtltl
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#150	STQDLYDNPVTAVF

Fig. 15

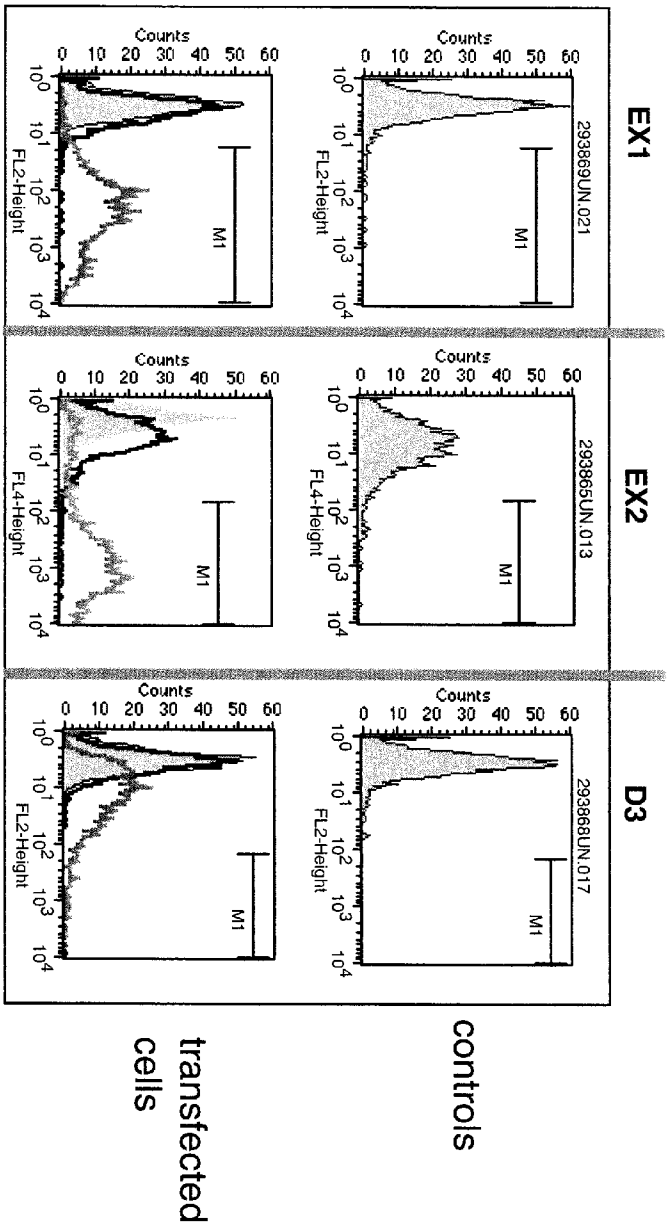

extracellular
intracellular
N
C
1
2
3
EX1
EX2
D3
Counts
FL2-Height
FL4-Height
M1
293869UN.021
293865UN.013
293868UN.017
controls
transfected cells

Fig. 16

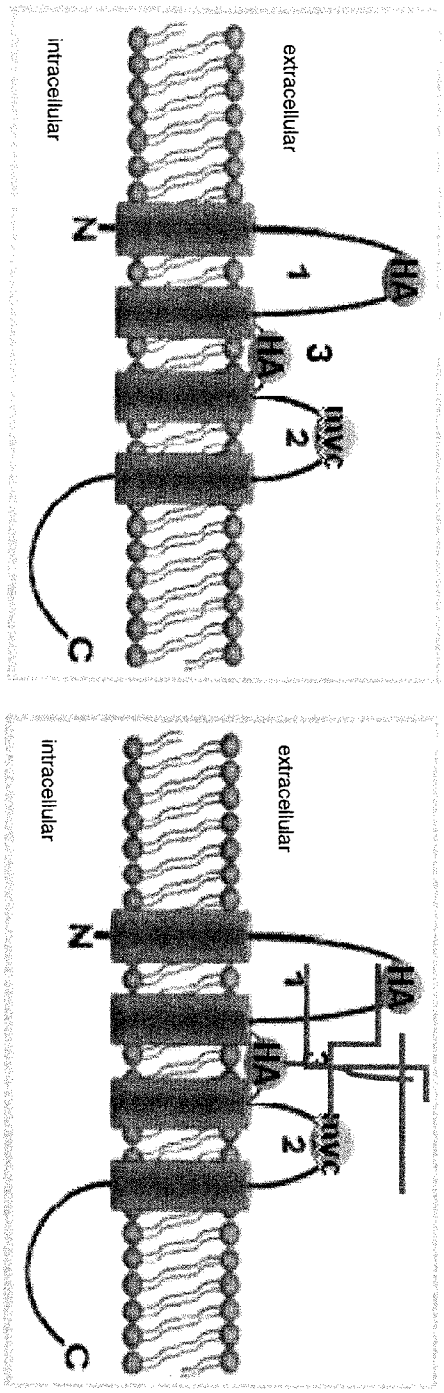
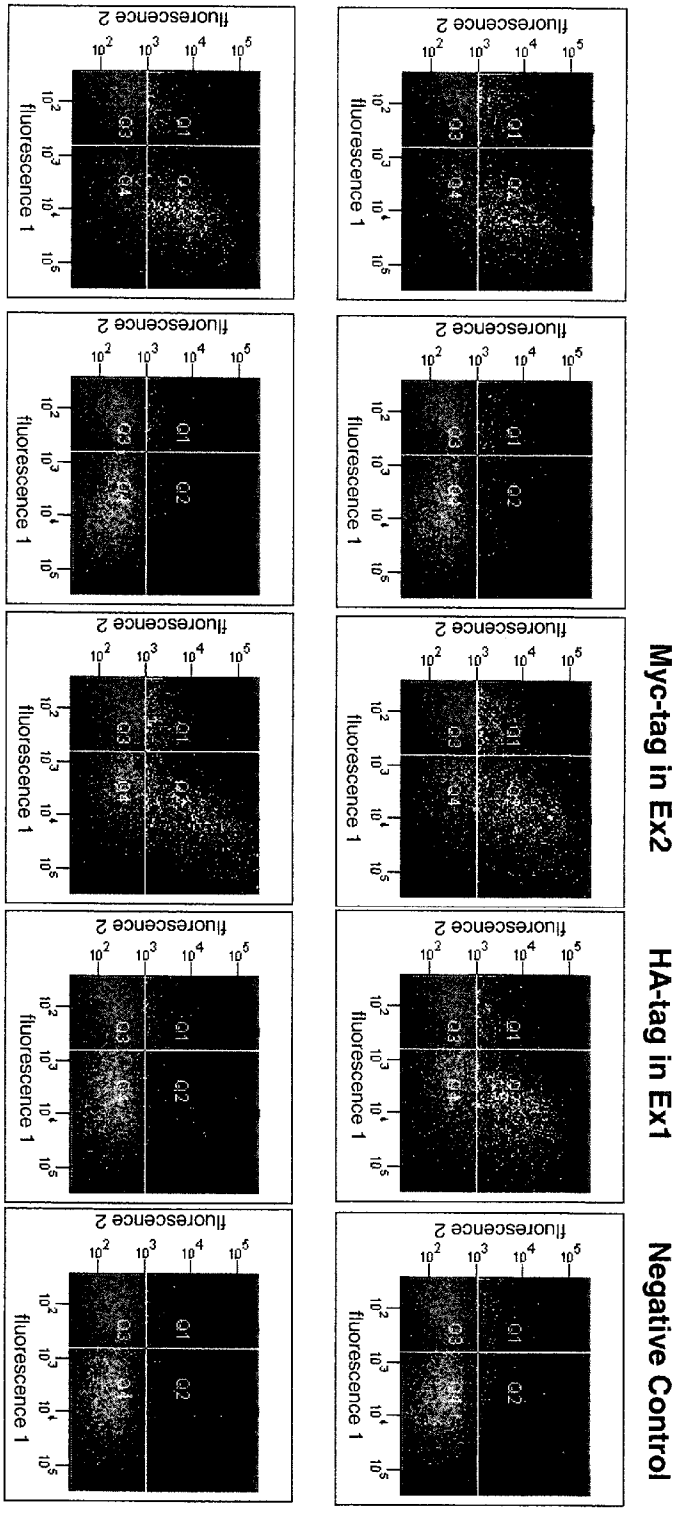

cancer
extracellular
intracellular
N
C
HA
HA
HA
mVC
1
2
3
normal tissues
extracellular
intracellular
N
C
HA
HA
HA
mVC
1
2
3

Fig. 17


mAB1
mAB2
Claudin 18A2
Claudin 18A1
Claudin 18A2 Myc-tag in Ex2
Claudin 18A2 HA-tag in Ex1
Mock Negative Control
fluorescence 1
fluorescence 2
Q1
Q2
Q3
Q4