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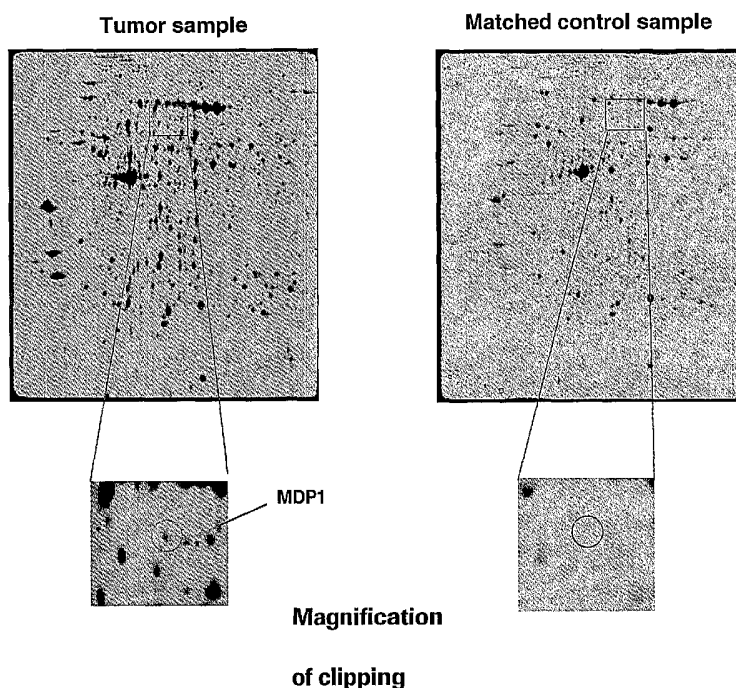
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(54) Title: USE OF MICROSOMAL DIPEPTIDASE AS A MARKER FOR COLORECTAL CANCER



(57) Abstract: The present invention relates to the diagnosis of colorectal cancer. It discloses the use of protein MDP 1 in the diagnosis of colorectal cancer. It relates to a method for diagnosis of colorectal cancer from a liquid sample, derived from an individual by measuring MDP1 in said sample. Measurement of MDP1 can, e.g., be used in the early detection or diagnosis of colorectal cancer.



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USE OF MICROSOMAL DIPEPTIDASE AS A MARKER FOR COLORECTAL CANCER

The present invention relates to the diagnosis of colorectal cancer. It discloses the use of MDP1 (= dehydropeptidase-I) in the diagnosis of colorectal cancer. Furthermore, it especially relates to a method for diagnosis of colorectal cancer from a liquid sample, derived from an individual by measuring MDP1 in said sample. Measurement of MDP1 can, e.g., be used in the early detection of colorectal cancer or in the surveillance of patients who undergo surgery.

Cancer remains a major public health challenge despite progress in detection and therapy. Amongst the various types of cancer, colorectal cancer (= CRC) is one of the most frequent cancers in the Western world.

Colorectal cancer most frequently progresses from adenomas (polyps) to malignant carcinomas. The different stages of CRC used to be classified according to Dukes' stages A to D.

The staging of cancer is the classification of the disease in terms of extent, progression, and severity. It groups cancer patients so that generalizations can be made about prognosis and the choice of therapy.

Today, the TNM system is the most widely used classification of the anatomical extent of cancer. It represents an internationally accepted, uniform staging system. There are three basic variables: T (the extent of the primary tumor), N (the status of regional lymph nodes) and M (the presence or absence of distant metastases). The TNM criteria are published by the UICC (International Union Against Cancer), edition, 1997 (Sobin, L.H., and Fleming, I.D., TNM 80 (1997) 1803-4)

What is especially important is, that early diagnosis of CRC translates to a much better prognosis. Malignant tumors of the colorectum arise from benign tumors, i.e. from adenoma. Therefore, best prognosis have those patients diagnosed at the adenoma stage. Patients diagnosed as early as in stage T_{is}, N0, M0 or T1-3; N0; M0, if treated properly have a more than 90% chance of survival 5 years after diagnosis as compared to a 5-years survival rate of only 10% for patients diagnosed when distant metastases are already present.

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In the sense of the present invention early diagnosis of CRC refers to a diagnosis at a pre-malignant state (adenoma) or at a tumor stage where no metastases at all (neither proximal nor distal), i.e., adenoma, T_{is}, N0, M0 or T1-4; N0; M0 are present. T_{is} denotes carcinoma *in situ*.

- 5 It is further preferred, that CRC is diagnosed when it has not yet fully grown through the bowel wall and thus neither the visceral peritoneum is perforated nor other organs or structures are invaded, i.e., that diagnosis is made at stage T_{is}, N0, M0 or T1-3; N0; M0 (=T_{is}-3; N0; M0).

- 10 The earlier cancer can be detected/diagnosed, the better is the overall survival rate. This is especially true for CRC. The prognosis in advanced stages of tumor is poor. More than one third of the patients will die from progressive disease within five years after diagnosis, corresponding to a survival rate of about 40% for five years. Current treatment is only curing a fraction of the patients and clearly has the best effect on those patients diagnosed in an early stage of disease.

- 15 With regard to CRC as a public health problem, it is essential that more effective screening and preventative measures for colorectal cancer be developed.

- 20 The earliest detection procedures available at present for colorectal cancer involve using tests for fecal blood or endoscopic procedures. However, significant tumor size must typically exist before fecal blood is detected. The sensitivity of the guaiac-based fecal occult blood tests is ~26%, which means 74% of patients with malignant lesions will remain undetected (Ahlquist, D.A., Gastroenterol. Clin. North Am. 26 (1997) 41-55). The visualization of precancerous and cancerous lesions represents the best approach to early detection, but colonoscopy is invasive with significant costs, risks, and complications (Silvis, S.E., et al., JAMA 235 (1976) 928-930; 25 Geenen, J.E., et al., Am. J. Dig. Dis. 20 (1975) 231-235; Anderson, W.F., et al., J. Natl. Cancer Institute 94 (2002) 1126-1133).

- 30 In order to be of clinical utility a new diagnostic marker as a single marker should be at least as good as the best single marker known in the art. Or, a new marker should lead to a progress in diagnostic sensitivity and/or specificity either if used alone or in combination with one or more other markers, respectively. The

diagnostic sensitivity and/or specificity of a test is best assessed by its receiver-operating characteristics, which will be described in detail below.

5 The clinical utility of biochemical markers in colorectal cancer has recently been reviewed by the European Group on Tumor Markers (EGTM) (Duffy, M.J., et al Europ. J. of Cancer 39 (2003) 718-727).

At present, primarily diagnostic blood tests based on the detection of carcinoembryonic antigen (CEA), a tumor-associated glycoprotein, are available to assist diagnosis in the field of CRC. CEA is increased in 95% of tissue samples obtained from patients with colorectal, gastric, and pancreatic cancers and in the majority of breast, lung, and head and neck carcinomas (Goldenberg, D.M., et al., J. Natl. Cancer Inst. (Bethesda) 57 (1976) 11-22). Elevated CEA levels have also been reported in patients with nonmalignant disease, and many patients with colorectal cancer have normal CEA levels in the serum, especially during the early stage of the disease (Carriquiry, L.A., and Pineyro, A., Dis. Colon Rectum 42 (1999) 921-929; Herrera, M.A., et al., Ann. Surg. 183 (1976) 5-9; Wanebo, H.J., et al., N. Engl. J. Med. 299 (1978) 448-451). The utility of CEA as measured from serum or plasma in detecting recurrences is reportedly controversial and has yet to be widely applied (Martell, R.E., et al., Int. J. Biol. Markers 13 (1998) 145-149; Moertel, C.G., et al., JAMA 270 (1993) 943-947).

20 In light of the available data, serum CEA determination possesses neither the sensitivity nor the specificity to enable its use as a screening test for colorectal cancer in the asymptomatic population (Reynoso, G., et al., JAMA 220 (1972) 361-365; Sturgeon, C., Clinical Chemistry 48 (2002) 1151-1159).

25 Whole blood, serum or plasma are the most widely used sources of sample in clinical routine. The identification of an early CRC tumor marker that would aid in the reliable cancer detection or provide early prognostic information could lead to a diagnostic assay that would greatly aid in the diagnosis and in the management of this disease. Therefore, an urgent clinical need exists to improve the *in vitro* assessment of CRC. It is especially important to improve the early diagnosis of CRC, since for patients diagnosed early on chances of survival are much higher as compared to those diagnosed at a progressed stage of disease.

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It was the task of the present invention to investigate whether a biochemical marker can be identified which may be used in assessing CRC.

Surprisingly, it has been found that use of the marker MDP1 can at least partially overcome the problems known from the state of the art.

5 The present invention therefore relates to a method for assessing colorectal cancer *in vitro* by one or more biochemical markers comprising a) measuring in a sample the concentration of MDP1, and b) using the concentration determined in step (a) in the assessment of colorectal cancer.

10 Another preferred embodiment of the invention is a method for assessing colorectal cancer comprising the steps of a) contacting a liquid sample obtained from an individual with a specific binding agent for MDP1 under conditions appropriate for formation of a complex between said binding agent and MDP1, and b) correlating the amount of complex formed in (a) to the assessment of colorectal cancer.

15 Yet another preferred embodiment of the invention relates to a method for assessing colorectal cancer *in vitro* by biochemical markers, comprising measuring in a sample the concentration of MDP1 and of one or more other marker of colorectal cancer and using the concentrations determined in the assessment of colorectal cancer.

20 The present invention also relates to the use of a marker panel comprising at least MDP1 and CYFRA 21-1 in the assessment of CRC.

The present invention also relates to the use of a marker panel comprising at least MDP1 and NSE in the assessment of CRC.

The present invention also relates to the use of a marker panel comprising at least MDP1 and CEA in the assessment of CRC.

25 The present invention also relates to the use of a marker panel comprising at least MDP1 and NNMT in the assessment of CRC.

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The present invention also relates to the use of a marker panel comprising at least MDP1 and CA 19-9 in the assessment of CRC.

The present invention also relates to the use of a marker panel comprising at least MDP1 and CA 72-4 in the assessment of CRC.

5 The present invention also provides a kit for performing the method according to the present invention comprising at least the reagents required to specifically measure MDP1 and CYFRA 21-1, respectively, and optionally auxiliary reagents for performing the measurement.

10 The present invention also provides a kit for performing the method according to the present invention comprising at least the reagents required to specifically measure MDP1 and NSE, respectively, and optionally auxiliary reagents for performing the measurement.

15 In a further preferred embodiment the present invention relates to a method for assessing colorectal cancer *in vitro* comprising the steps of a) measuring in a sample the concentration of MDP1, b) optionally measuring in the sample the concentration of one or more other marker of colorectal cancer, and c) using the concentrations determined in step (a) and optionally step (b) in the assessment of colorectal cancer.

20 In a further preferred embodiment the present invention relates to a method of assessing colorectal cancer *in vitro* by one or more biochemical markers, comprising the steps of (a) providing a colonic tissue sample, (b) detecting MDP1 in the tissue sample, and (c) using the result of step (b) in the assessment of colorectal cancer.

As used herein, each of the following terms has the meaning associated with it in this section.

25 The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "a marker" means one marker or more than one marker.

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The term "marker" or "biochemical marker" as used herein refers to a molecules to be used as a target for analyzing patient test samples. Examples of such molecular targets are proteins or polypeptides themselves as well as antibodies present in a sample. Proteins or polypeptides used as a marker in the present invention are contemplated to include any variants of said protein as well as fragments of said protein or said variant, in particular, immunologically detectable fragments. One of skill in the art would recognize that proteins which are released by cells or present in the extracellular matrix which become damaged, e.g., during inflammation could become degraded or cleaved into such fragments. Certain markers are synthesized in an inactive form, which may be subsequently activated by proteolysis. As the skilled artisan will appreciate, proteins or fragments thereof may also be present as part of a complex. Such complex also may be used as a marker in the sense of the present invention. Variants of a marker polypeptide are encoded by the same gene, but differ in their PI or MW, or both (e.g., as a result of alternative mRNA or pre-mRNA processing, e.g. alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (e.g., glycosylation, acylation, and/or phosphorylation).

The term "assessing colorectal cancer" is used to indicate that the method according to the present invention will (alone or together with other markers or variables, e.g., the criteria set forth by the UICC (UICC (International Union Against Cancer), Sobin, L.H., Wittekind, Ch. (eds), TNM Classification of Malignant Tumours, fifth edition, 1997)) e.g., aid the physician to establish or confirm the absence or presence of CRC or aid the physician in the prognosis, the detection of recurrence (follow-up of patients after surgery) and/or the monitoring of treatment, especially of chemotherapy.

The term "sample" as used herein refers to a biological sample obtained for the purpose of evaluation *in vitro*. In the methods of the present invention, the sample or patient sample preferably may comprise any body fluid. Preferred test samples include blood, serum, plasma, urine, saliva, and synovial fluid. Preferred samples are whole blood, serum, plasma or synovial fluid, with plasma or serum being most preferred. As the skilled artisan will appreciate, any such assessment is made *in vitro*. The patient sample is discarded afterwards. The patient sample is solely used for the *in vitro* method of the invention and the material of the patient sample is

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not transferred back into the patient's body. Typically, the sample is a liquid sample, e.g., whole blood, serum, or plasma.

5 In a preferred embodiment the present invention relates to a method for assessing CRC *in vitro* by biochemical markers, comprising measuring in a sample the concentration of MDP1 and using the concentration determined in the assessment of CRC.

10 The protein MDP1 (DPEP1, MDP, dehydropeptidase-I, renal dipeptidase, RDP) (Swiss-PROT: P16444) is characterized by the sequence given SEQ ID NO:1. MDP1 is a zinc-dependent metallopeptidase that hydrolyzes a variety of dipeptides and is involved in glutathione metabolism (Kozak, E.M. and Tate, S.S., J. Biol. Chem. 257 (1982) 6322-6327). The protein begins with a 16 amino acid signal peptide, carries four potential N-glycosylation sites, and is anchored to the membrane by a covalently attached glycosyl-phosphatidylinositol moiety (Hooper, N.M., et al., Biochem. J. 244 (1987) 465-469; Adachi, H., et al., J. Biol. Chem. 265 (1990) 3992-15 3995). The deduced size of the unprocessed precursor peptide backbone is 46 kDa. Its major site of expression is the epithelial cells of the proximal tubules of the kidney.

20 MDP1 was identified as a potential tumor suppressor gene due to its decreased expression in Wilms' tumor when compared to normal kidney (Austruy, E., et al Cancer Res. 53 (1993) 2888-2894). In contrast, other expression studies have shown this gene to be over-expressed in colorectal neoplasms compared to normal mucosa. The most comprehensive study on the expression in colon tumor and normal tissue is reported by McIver, C.M., et al., Cancer Letters 209 (2004) 67-74, where determining the DPEP1 RNA level by RT-PCR was suggested as a marker of 25 disseminated tumor cells.

30 As obvious to the skilled artisan, the present invention shall not be construed to be limited to the full-length protein MDP1 of SEQ ID NO: 1. Physiological or artificial fragments of MDP1, secondary modifications of MDP1, as well as allelic variants of MDP1 are also encompassed by the present invention. Artificial fragments preferably encompass a peptide produced synthetically or by recombinant techniques, which at least comprises one epitope of diagnostic interest consisting of at least 6 contiguous amino acids as derived from the sequence disclosed in SEQ ID

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5 NO:1. Such fragment may advantageously be used for generation of antibodies or as a standard in an immunoassay. More preferred the artificial fragment comprises at least two epitopes of interest appropriate for setting up a sandwich immunoassay. Preferably, full-length MDP1 or a physiological variant is detected in a method according to the present invention,

The assessment method according to the present invention is based on a liquid sample which is derived from an individual. Unlike to methods known from the art MDP1 is specifically measured from this liquid sample by use of a specific binding agent.

10 A specific binding agent is, e.g., a receptor for MDP1, a lectin binding to MDP1 or an antibody to MDP1. A specific binding agent has at least an affinity of 10^7 l/mol for its corresponding target molecule. The specific binding agent preferably has an affinity of 10^8 l/mol or even more preferred of 10^9 l/mol for its target molecule. As the skilled artisan will appreciate the term specific is used to indicate that other
15 biomolecules present in the sample do not significantly bind to the binding agent specific for MDP1. Preferably, the level of binding to a biomolecule other than the target molecule results in a binding affinity which is only 10%, more preferably only 5% of the affinity of the target molecule or less. A most preferred specific binding agent will fulfill both the above minimum criteria for affinity as well as for
20 specificity.

A specific binding agent preferably is an antibody binding to MDP1. The term antibody refers to a polyclonal antibody, a monoclonal antibody, fragments of such antibodies, as well as to genetic constructs comprising the binding domain of an antibody.

25 Any antibody fragment retaining the above criteria of a specific binding agent can be used. Antibodies are generated by state of the art procedures, e.g., as described in Tijssen (Tijssen, P., Practice and theory of enzyme immunoassays 11 (1990) the whole book, especially pages 43-78; Elsevier, Amsterdam). In addition, the skilled artisan is well aware of methods based on immunosorbents that can be used for the
30 specific isolation of antibodies. By these means the quality of polyclonal antibodies and hence their performance in immunoassays can be enhanced. (Tijssen, P., *supra*, pages 108-115).

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For the achievements as disclosed in the present invention polyclonal antibodies raised in rabbits have been used. However, clearly also polyclonal antibodies from different species, e.g. rats or guinea pigs, as well as monoclonal antibodies can also be used. Since monoclonal antibodies can be produced in any amount required
5 with constant properties, they represent ideal tools in development of an assay for clinical routine. The generation and use of monoclonal antibodies to MDP1 in a method according to the present invention is yet another preferred embodiment.

As the skilled artisan will appreciate now, that MDP1 has been identified as a marker which is useful in the diagnosis of CRC, alternative ways may be used to
10 reach a result comparable to the achievements of the present invention. For example, alternative strategies to generate antibodies may be used. Such strategies comprise amongst others the use of synthetic peptides, representing an epitope of MDP1 for immunization. Alternatively, DNA Immunization also known as DNA vaccination may be used.

15 For measurement the liquid sample obtained from an individual is incubated with the specific binding agent for MDP1 under conditions appropriate for formation of a binding agent MDP1-complex. Such conditions need not be specified, since the skilled artisan without any inventive effort can easily identify such appropriate incubation conditions.

20 As a final step according to the method disclosed in the present invention the amount of complex is measured and correlated to the diagnosis of CRC. As the skilled artisan will appreciate there are numerous methods to measure the amount of the specific binding agent MDP1-complex all described in detail in relevant textbooks (cf., e.g., Tijssen P., *supra*, or Diamandis, et al., eds. (1996) Immunoassay,
25 Academic Press, Boston).

Preferably MDP1 is detected in a sandwich type assay format. In such assay a first specific binding agent is used to capture MDP1 on the one side and a second specific binding agent, which is labeled to be directly or indirectly detectable is used on the other side.

30 As shown on Figures 1 and 2 MDP1 can be detected in a cancerous tissue sample, e.g. by means of a Western Blot following homogenization of the sample. However,

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another preferred embodiment of the invention is detecting MDP1 in the tissue sample *in situ*. To this end, the skilled artisan is aware of histological methods (Elias, J.M. (2003) Immunohistopathology: A Practical Approach to Diagnosis (2nd edition) American Society Clinical Pathology) using labeled substances which target
5 MDP1 in the tissue sample in order to identify MDP1 on a microscopic level. Preferably, MDP1 is analyzed qualitatively, e.g. its occurrence in particular cell types, its intracellular localization or its localization in extracellular compartments or spaces. Also preferred, MDP1 is analyzed quantitatively, e.g. using immunofluorescence and quantification of fluorescence intensity. In a further
10 preferred embodiment MDP1 is detected *in situ* together with one or more other cytological or biochemical markers.

As mentioned above, it has surprisingly been found that MDP1 can be measured from a liquid sample obtained from an individual sample. No tissue and no biopsy sample is required to apply the marker MDP1 in the assessment of CRC.

15 In a preferred embodiment the method according to the present invention is practiced with serum as liquid sample material. In a further preferred embodiment the method according to the present invention is practiced with plasma as liquid sample material. In a further preferred embodiment the method according to the present invention is practiced with whole blood as liquid sample material.

20 Furthermore stool can be prepared in various ways known to the skilled artisan to result in a liquid sample as well. Such sample liquid derived from stool also represents a preferred embodiment according to the present invention.

The inventors of the present invention have surprisingly been able to detect protein MDP1 in a bodily fluid sample. Even more surprising they have been able to
25 demonstrate that the presence of MDP1 in such liquid sample obtained from an individual can be correlated to the assessment of colorectal cancer. Preferably, an antibody to MDP1 is used in a qualitative (MDP1 present or absent) or quantitative (MDP1 amount is determined) immunoassay.

Measuring the level of protein MDP1 has proven very advantageous in the field of
30 CRC. Therefore, in a further preferred embodiment, the present invention relates

to use of protein MDP1 as a marker molecule in the assessment of colorectal cancer from a liquid sample obtained from an individual.

5 The ideal scenario for diagnosis would be a situation wherein a single event or process would cause the respective disease as, e.g., in infectious diseases. In all other cases correct diagnosis can be very difficult, especially when the etiology of the disease is not fully understood as is the case of CRC. As the skilled artisan will appreciate, no biochemical marker, for example in the field of CRC, is diagnostic with 100% specificity and at the same time 100% sensitivity for a given disease. Rather, biochemical markers are used to assess with a certain likelihood or
10 predictive value the presence or absence of a disease. Therefore, in routine clinical diagnosis various clinical symptoms and biological markers are generally considered together in the diagnosis, treatment, and management of the underlying disease.

15 Biochemical markers can either be determined individually or, in a preferred embodiment of the invention, they can be measured simultaneously using a chip- or a bead-based array technology. The concentrations of the biomarkers are then interpreted independently using an individual cut-off for each marker or they are combined for interpretation.

20 In a further preferred embodiment of the invention the assessment of colorectal cancer according to the present invention is performed in a method comprising the steps of a) measuring in a sample the concentration of MDP1, b) optionally measuring in the sample one or more other marker of colorectal cancer, and c) using the concentration determined in step (a) and optionally the concentration(s) determined in step (b) in the assessment of colorectal cancer.

25 Preferably the method for assessment of CRC is performed by measuring the concentration of MDP1 and of one or more other marker and by using the concentration of MDP1 and the concentration(s) of the one or more other marker in the assessment of CRC.

30 The present invention is also directed to a method for assessing CRC *in vitro* by biochemical markers, comprising measuring in a sample the concentration of MDP1 and of one or more other marker of CRC and using the concentrations determined in the assessment of CRC.

According to the data shown in the Example section the marker MDP1 in the univariate analysis has (at a specificity of about 90%) a sensitivity for CRC of 54.7%. In the assessment of CRC the marker MDP1 will be of advantage in one or more of the following aspects: screening; diagnostic aid; prognosis; monitoring of chemotherapy, and follow-up.

Screening:

CRC is the second most common malignancy of both males and females in developed countries. Because of its high prevalence, its long asymptomatic phase and the presence of premalignant lesions, CRC meets many of the criteria for screening. Clearly, a serum tumour marker which has acceptable sensitivity and specificity would be more suitable for screening than either FOB testing or endoscopy.

As the data given in the Examples section demonstrate MDP1 alone will not suffice to allow for a general screening e.g. of the at risk population for CRC. Most likely no single biochemical marker in the circulation will ever meet the sensitivity and specificity criteria required for screening purposes. Rather it has to be expected that a marker panel will have to be used in CRC screening. The data established in the present invention indicate that the marker MDP1 will form an integral part of a marker panel appropriate for screening purposes. The present invention therefore relates to the use of MDP1 as one marker of a CRC marker panel for CRC screening purposes. The present data further indicate that certain combinations of markers will be advantageous in the screening for CRC. Therefore the present invention also relates to the use of a marker panel comprising MDP1 and CYFRA 21-1, or of a marker panel comprising MDP1 and NSE, or of a marker panel comprising MDP1 and CYFRA 21-1 and NSE for the purpose of screening for CRC.

Diagnostic aid:

Preoperative CEA values are of limited diagnostic value. Nonetheless the European Committee on Tumor Markers (ECTM) recommends that CEA should be measured before surgery in order to establish a baseline value and for assessing the prognosis. Since MDP1 as a single marker according to the data of the present invention might be at least as good a single marker as CEA or even superior it has to

be expected that MDP1 will be used as a diagnostic aid, especially by establishing a baseline value before surgery.

The present invention thus also relates to the use of MDP1 for establishing a baseline value before surgery for CRC.

5 **Prognosis:**

10 The gold standard for determining prognosis in patients with CRC is the extend of disease as defined by the Dukes', TNM or other staging systems, If a marker such as CEA is to be used for predicting outcome, it must: provide stronger prognostic information than that offered by existing staging systems, provide information independent of the existing systems or provide prognostic data within specific subgroups defined by existing criteria, e.g. in Dukes' B or node-negative patients.

15 Recently, an American Joint Committee on Cancer (AJCC) Consensus Conference suggested that CEA should be added to the TNM staging system for colorectal cancer. The CEA level should be designated as follows: CX, CEA cannot be assessed; CO, CEA not elevated ($<5\mu\text{g/l}$) or CEA1, CEA elevated ($> 5\mu\text{g/l}$) (Compton, C., et al., Cancer 88 (2000) 1739-1757).

20 As MDP1 alone significantly contributes to the differentiation of CRC patients from healthy controls or from healthy controls plus non-malignant colon diseases, it has to be expected that it will aid in assessing the prognosis of patients suffering from CRC. The level of preoperative MDP1 will most likely be combined with one or more other marker for CRC and/or the TNM staging system, as recommended for CEA by the AJCC. In a preferred embodiment MDP1 is used in the prognosis of patients with CRC.

Monitoring of Chemotherapy:

25 A number of reports have described the use of CEA in monitoring the treatment of patients with advanced CRC (for review, see Refs. Duffy, M.J., Clin. Chem. 47 (2001) 625-630; Fletcher, R.H., Ann. Int. Med. 104 (1986) 66-73; Anonymous, J. Clin. Oncol. 14 (1996) 2843-2877). Most of these were retrospective, non-randomized and contained small numbers of patients. These studies suggested: a)

that patients with a decrease in CEA levels while receiving chemotherapy generally had a better outcome than those patients whose CEA levels failed to decrease and (b) for almost all patients, increases in CEA levels were associated with disease progression.

- 5 Due to the data shown in the example section, it has to be expected that MDP1 will be at least as good a marker for monitoring of chemotherapy as CEA. The present invention therefore also relates to the use of MDP1 in the monitoring of CRC patients under chemotherapy.

Follow-up:

- 10 Approximately 50 % of patients who undergo surgical resection aimed at cure, later develop recurrent or metastatic disease (Berman, J.M., et al., Lancet 355 (2000) 395-399). Most of these relapses occur within the first 2-3 years of diagnosis and are usually confined to the liver, lungs or locoregional areas. Since recurrent/metastatic disease is invariably fatal, considerable research has focused on its identification at
15 an early and thus potentially treatable stage. Consequently, many of these patients undergo a postoperative surveillance program which frequently includes regular monitoring with CEA.

- Serial monitoring with CEA has been shown to detect recurrent/metastatic disease with a sensitivity of approximately of 80 %, specificity of approximately 70 % and
20 provides an average lead-time of 5 months (for review, see Duffy, M.J., et al., supra, and Fletcher, R.H., supra). Furthermore, CEA was the most frequent indicator of recurrence in asymptomatic patients (Pietra, N., et al., Dis. Colon Rectum 41 (1998) 1127-1133 and Graham, R.A., et al., Ann Surg 228 (1998) 59-63) and was more cost-effective than radiology for the detection of potentially curable recurrent
25 disease. As regards sites of recurrence/metastasis, CEA was most sensitive (almost 100%) for the detection of liver metastasis. On the other hand, CEA was less reliable for diagnosing locoregional recurrences, the sensitivity being only approximately 60 % (Moertel, C.G., et al., Jama 270 (1993) 943-7)

- 30 As a compromise between patient convenience, costs and efficiency of disease detection, the EGTM Panel like the MDP1O Panel (Anonymous, J. Clin. Oncol. 14 (1996) 2843-2877) suggests that CEA testing be carried out every 2-3 months for at

least 3 years after the initial diagnosis. After 3 years, testing could be carried out less frequently, e.g. every 6 months. No evidence exists, however, to support this frequency of testing.

5 As the above discussion of the state of the art shows, that the follow-up of patients with CRC after surgery is one of the most important fields of use for an appropriate biochemical marker. Due to the high sensitivity of MDP1 in the CRC patients investigated it is expected that MDP1 alone or in combination with one or more other marker will be of great help in the follow-up of CRC patients, especially in CRC patients after surgery. The use of a marker panel comprising MDP1 and one
10 or more other marker of CRC in the follow-up of CRC patients represents a further preferred embodiment of the present invention.

The present invention discloses and therefore in a preferred embodiment relates to the use of MDP1 in the diagnostic field of CRC or in the assessment of CRC, respectively.

15 In yet a further preferred embodiment the present invention relates to the use of MDP1 as a marker molecule for colorectal cancer in combination with one or more marker molecules for colorectal cancer in the assessment of colorectal cancer from a liquid sample obtained from an individual. In this regard, the expression "one or more" denotes 1 to 20, preferably 1 to 10, preferably 1 to 5, more preferred 3 or 4.
20 MDP1 and the one or more other marker form a CRC marker panel.

Thus, a preferred embodiment of the present invention is the use of MDP1 as a marker molecule for colorectal cancer in combination with one or more marker molecules for colorectal cancer in the assessment of colorectal cancer from a liquid sample obtained from an individual. Preferred selected other CRC markers with
25 which the measurement of MDP1 may be combined are NSE, CYFRA 21-1, NMMT, CA 19-9, CA 72-4, and/or CEA. Yet further preferred the marker panel used in the assessment of CRC comprises MDP1 and at least one other marker molecule selected from the group consisting of NSE, CYFRA 21-1 and NMMT.

30 The markers which preferably are combined with MDP1 or which form part of the CRC marker panel comprising MDP1, respectively, are discussed in more detail below.

NSE (neuron-specific enolase)

The glycolytic enzyme enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11, molecular weight approx. 80 kD) occurs in a variety of dimeric isoforms comprising three immunologically different subunits termed α , β , and γ . The α -subunit of enolase occurs in numerous types of tissue in mammals, whereas the β -subunit is found mainly in the heart and in striated musculature. The enolase isoforms $\alpha\gamma$ and $\gamma\gamma$, which are referred to as neuron-specific enolase (NSE) or γ -enolase, are primarily detectable in high concentrations in neurons and neuro-endocrine cells as well as in tumors originating from them. (Lamerz R., NSE (Neuronen-spezifische Enolase), γ -Enolase. In: Thomas L (ed) Clinical Laboratory Diagnosis, TH-Books, Frankfurt, 1st English Edition 1998: 979-981, 5. deutsche Auflage 1998:1000-1003)

NSE is described as the marker of first choice in the monitoring of small cell bronchial carcinoma, (Lamerz R., *supra*), whereas CYFRA 21-1 is superior to NSE for non-small cell bronchial carcinoma. (Ebert W., et al., Eur. J. Clin. Chem. Clin. Biochem 32 (1994) 189-199).

Elevated NSE concentrations are found in 60-81 % of cases of small cell bronchial carcinoma.

For NSE there is no correlation to the site of metastasis or to cerebral metastasis, but there is good correlation to the clinical stage, i.e. the extent of the disease.

In response to chemotherapy there is a temporary rise in the NSE level 24-72 hours after the first therapy cycle as a result of cytolysis of the tumor cells. This is followed within a week or by the end of the first therapy cycle by a rapid fall in the serum values (which were elevated prior to therapy). By contrast, non-responders to therapy display levels which are constantly elevated or fail to fall into the reference range. During remission, 80-96 % of the patients have normal values. Rising NSE values are found in cases of relapse. The rise occurs in some cases with a latent period of 1-4 months, is often exponential (with a doubling time of 10-94 days) and correlates with the survival period. NSE is useful as a single prognostic factor and activity marker during the monitoring of therapy and the course of the disease in

small cell bronchial carcinoma: diagnostic sensitivity 93 %, positive predictive value 92% (Lamerz R., *supra*)

5 In neuroblastoma NSE serum values above 30 ng/ml are found in 62 % of the affected children. The medians rise in accordance with the stage of the disease. There is a significant correlation between the magnitude or frequency of pathological NSE values and the stage of disease; there is an inverse correlation with illness-free survival.

10 68-73 % of the patients with seminoma have a clinically significant NSE elevation. (Lamerz R., *supra*). There is a utilizable correlation with the clinical course of the disease.

15 NSE has also been measured in other tumors: Non-pulmonary malignant diseases show values above 25 ng/ml in 22 % of the cases (carcinomas in all stages). Brain tumors such as glioma, meningioma, neurofibroma, and neurinoma are only occasionally accompanied by elevated serum NSE values. In primary brain tumors or brain metastasis and in malignant melanoma and pheochromocytoma, elevated NSE-values can occur in the CSF (cerebrospinal fluid). Increased NSE concentrations have been reported for 14 % of organ-restricted and 46 % of metastasizing renal carcinomas, with a correlation to the grade as an independent prognosis factor.

20 In benign disease elevated serum NSE concentrations (> 12 ng/ml) have been found in patients with benign pulmonary diseases and cerebral diseases. Elevated values, mainly in the liquor, have been found in cerebrovascular meningitis, disseminated encephalitis, spinocerebellar degeneration, cerebral ischemia, cerebral infarction, intracerebral hematoma, subarachnoid hemorrhage, head injuries, inflammatory
25 brain diseases, organic epilepsy, schizophrenia, and Jakob-Creutzfeldt disease. (Lamerz R., *supra*)

NSE has been measured on an Elecsys® analyzer using Roche product number 12133113 according to the manufacturers instructions.

CA 19-9 Carbohydrate Antigen 19-9

5 The CA 19-9 values measured are defined by the use of the monoclonal antibody 1116-NS-19-9. The 1116-NS-19-9-reactive determinants on a glycolipid having a molecular weight of approx. 10,000 daltons are measured. This mucin corresponds to a hapten of Lewis-a blood group determinants and is a component of a number of mucous membrane cells. (Koprowski, H., et al., Somatic Cell Genet 5 (1979) 957-971).

10 3-7 % of the population have the Lewis a-negative/b-negative blood group configuration and are unable to express the mucin with the reactive determinant CA 19-9. This must be taken into account when interpreting the findings.

15 Mucin occurs in fetal gastric, intestinal and pancreatic epithelia. Low concentrations can also be found in adult tissue in the liver, lungs, and pancreas. (Stieber, P. and Fateh-Moghadam, A., Boeringer Mannheim, Cat. No. 1536869 (engl), 1320947 (dtsh), ISBN 3-926725-07-9 dtsh/engl., Juergen Hartmann Verlag, Marloffstein-Rathsberg (1993); Herlyn, M., et al., J. Clin. Immunol 2 (1982) 135-140).

20 CA 19-9 assay values can assist in the differential diagnosis and monitoring of patients with pancreatic carcinoma (sensitivity 70-87 %). (Ritts, R.E., Jr., et al., Int. J. Cancer 33 (1984) 339-345). There is no correlation between tumor mass and the CA 19-9 assay values. However, patients with CA 19-9 serum levels above 10,000 U/mL almost always have distal metastasis.

The determination of CA 19-9 cannot be used for the early detection of pancreatic carcinoma. (Steinberg, W.M., et al., Gastroenterology 90 (1986) 343-349).

25 In hepatobiliary carcinoma the CA 19-9 values provide a sensitivity of 50-75 %. The concomitant determination of CA 72-4 and CEA is recommended in case of gastric carcinoma. In colorectal carcinoma, determination of CEA alone is adequate; only in rare CEA-negative cases the determination of CA 19-9 can be useful.

As the mucin is excreted exclusively via the liver, even slight cholestasis can lead to clearly elevated CA 19-9 serum levels in some cases. Elevated CA 19-9 values are

also found with a number of benign and inflammatory diseases of the gastrointestinal tract and the liver, as well as in cystic fibrosis.

CA 19-9 has been measured on an Elecsys® analyzer using Roche product number 11776193 according to the manufacturers instructions.

5 CEA Carcinoembryonic antigen

CEA is a monomeric glycoprotein (molecular weight approx. 180.000 dalton) with a variable carbohydrate component of approx. 45-60 % (Gold, P. and Freedman S.O., J. Exp. Med. 121 (1965) 439-462).

10 CEA, like AFP, belongs to the group of carcino-fetal antigens that are produced during the embryonic and fetal period. The CEA gene family consists of about 17 active genes in two subgroups. The first group contains CEA and the Non-specific Cross-reacting Antigens (NCA); the second group contains the Pregnancy-Specific Glycoproteins (PSG).

15 CEA is mainly found in the fetal gastrointestinal tract and in fetal serum. It also occurs in slight quantities in intestinal, pancreatic, and hepatic tissue of healthy adults. The formation of CEA is repressed after birth, and accordingly serum CEA values are hardly measurable in healthy adults.

20 High CEA concentrations are frequently found in cases of colorectal adenocarcinoma (Stieber, P. and Fateh-Moghadam, A., *supra*). Slight to moderate CEA elevations (rarely > 10 ng/mL) occur in 20-50 % of benign diseases of the intestine, the pancreas, the liver, and the lungs (e.g. liver cirrhosis, chronic hepatitis, pancreatitis, ulcerative colitis, Crohn's Disease, emphysema) (Stieber, P. and Fateh-Moghadam, A., *supra*). Smokers also have elevated CEA values.

25 The main indication for CEA determinations is the follow-up and therapy management of colorectal carcinoma.

CEA determinations are not recommended for cancer-screening in the general population. CEA concentrations within the normal range do not exclude the possible presence of a malignant disease.

- 20 -

The antibodies in assay manufactured by Roche Diagnostics react with CEA and (as with almost all CEA methods) with the meconium antigen (NCA2). Cross-reactivity with NCA1 is 0.7 % (Hammarstrom ,S., et al., Cancer Res. 49 (1989) 4852-4858, and Bormer, O.P., Tumor Biol. 12 (1991) 9-15)

- 5 CEA has been measured on an Elecsys® analyzer using Roche product number 11731629 according to the manufacturers instructions.

CYFRA 21-1

- 10 An assay for "CYFRA 21-1" specifically measures a soluble fragment of cytokeratin 19 as present in the circulation. The measurement of CYFRA 21-1 is typically based upon two monoclonal antibodies (Bodenmueller, H., et al., Int. J. Biol. Markers 9 (1994) 75-81). In the CYFRA 21-1 assay from Roche Diagnostics, Germany, the two specific monoclonal antibodies (KS 19.1 and BM 19.21) are used and a soluble fragment of cytokeratin 19 having a molecular weight of approx. 30,000 daltons is measured.

- 15 Cytokeratins are structural proteins forming the subunits of epithelial intermediary filaments. Twenty different cytokeratin polypeptides have so far been identified. Due to their specific distribution patterns they are eminently suitable for use as differentiation markers in tumor pathology. Intact cytokeratin polypeptides are poorly soluble, but soluble fragments can be detected in serum. (Bodenmueller, H.,
20 et al., supra).

CYFRA 21-1 is a well-established marker for Non-Small-Cell Lung Carcinoma (NSCLC). The main indication for CYFRA 21-1 is monitoring the course of non-small cell lung cancer (NSCLC). (Sturgeon, C., Clinical Chemistry 48 (2002) 1151-1159).

- 25 High CYFRA 21-1 serum levels indicate an advanced tumor stage and a poor prognosis in patients with non-small-cell lung cancer. (van der Gaast A. et al., Br. J. Cancer 69 (1994) 525-528). A normal or only slightly elevated value does not rule out the presence of a tumor.

Successful therapy is documented by a rapid fall in the CYFRA 21-1 serum level into the normal range. A constant CYFRA 21-1 value or a slight or only slow decrease in the CYFRA 21-1 value indicates incomplete removal of a tumor or the presence of multiple tumors with corresponding therapeutic and prognostic consequences. Progression of the disease is often shown earlier by increasing CYFRA 21-1 values than by clinical symptomatology and imaging procedures.

It is accepted that in the primary diagnosis of pulmonary carcinoma should be made on the basis of clinical symptomatology, imaging or endoscopic procedures and intraoperative findings. An unclear circular focus in the lung together with CYFRA 21-1 values > 30 ng/mL indicates with high probability the existence of primary bronchial carcinoma.

CYFRA 21-1 is also suitable for course-monitoring in myoinvasive cancer of the bladder. Good specificity is shown by CYFRA 21-1 relative to benign lung diseases (pneumonia, sarcoidosis, tuberculosis, chronic bronchitis, bronchial asthma, emphysema).

Slightly elevated values (up to 10 ng/mL) are rarely found in marked benign liver diseases and renal failure. There is no correlation with sex, age or smoking. The values for CYFRA 21-1 are also unaffected by pregnancy.

Recently it has been found that CYFRA 21-1 also is of use in detecting disease relapse and assessing treatment efficacy in the field of breast cancer (Nakata, B., et al., British J. of Cancer (2004) 1-6).

CYFRA 21-1 has been measured on an Elecsys® analyzer using Roche product number 11820966 according to the manufacturers instructions.

As mentioned further above CYFRA 21-1 is an established marker in the field of NSCLC. When developing and establishing CYFRA 21-1 for NSCLC, non-malignant disease controls derived from patients with certain lung non-malignant diseases have been used. This has been considered important to differentiate benign from malign lung diseases (H. Bodenmüller, et al., *supra*).

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Since only recently it is possible to detect the marker CYFRA 21-1 in a significant percentage of samples derived from patients with CRC. In addition, the presence of CYFRA 21-1 in such liquid sample obtained from an individual can be used in the assessment of colorectal cancer. Particularly in combination with other markers
5 CYFRA 21-1 is considered to be a very useful marker in the field of CRC.

NNMT

The protein nicotinamide N-methyltransferase (NNMT; Swiss-PROT: P40261) has an apparent molecular weight of 29.6 kDa and an isoelectric point of 5.56.

NNMT catalyzes the N-methylation of nicotinamide and other pyridines. This
10 activity is important for biotransformation of many drugs and xenobiotic compounds. The protein has been reported to be predominantly expressed in liver and is located in the cytoplasm. NNMT has been cloned from cDNA from human liver and contained a 792-nucleotide open reading frame that encoded a 264-amino
15 acid protein with a calculated molecular mass of 29.6 kDa. (Aksoy, S., et al., J. Biol. Chem. 269 (1994) 14835-14840). Little is known in the literature about a potential role of the enzyme in human cancer. In one paper, increased hepatic NNMT
enzymatic activity was reported as a marker for cancer cachexia in mice (Okamura, A., et al., Jpn. J. Cancer Res. 89 (1998) 649-656). In a recent report, down-
20 regulation of the NNMT gene in response to radiation in radiation sensitive cell lines was demonstrated (Kassem, H., et al., Int. J. Cancer 101 (2002) 454-460).

It has recently been found (WO 2004/057336) that NNMT will be of interest in the assessment of CRC. The immunoassay described in WO 2004/057336 has been used to measure the samples (CRC, healthy controls and non-malignant colon diseases) of the present study.

25 As the skilled artisan will appreciate there are many ways to use the measurements of two or more markers in order to improve the diagnostic question under investigation. In a quite simple, but nonetheless often effective approach, a positive result is assumed if a sample is positive for at least one of the markers investigated. This may e.g. the case when diagnosing an infectious disease, like AIDS.

Frequently, however, the combination of markers is evaluated. Preferably the values measured for markers of a marker panel, e.g. for MDP1, CYFRA 21-1 and NSE, are mathematically combined and the combined value is correlated to the underlying diagnostic question. Marker values may be combined by any appropriate state of the art mathematical method. Well-known mathematical methods for correlating a marker combination to a disease employ methods like, discriminant analysis (DA) (i.e. linear-, quadratic-, regularized-DA), Kernel Methods (i.e. SVM), Nonparametric Methods (i.e. k-Nearest-Neighbor Classifiers), PLS (Partial Least Squares), Tree-Based Methods (i.e. Logic Regression, CART, Random Forest Methods, Boosting/Bagging Methods), Generalized Linear Models (i.e. Logistic Regression), Principal Components based Methods (i.e. SIMCA), Generalized Additive Models, Fuzzy Logic based Methods, Neural Networks and Genetic Algorithms based Methods. The skilled artisan will have no problem in selecting an appropriate method to evaluate a marker combination of the present invention. Preferably the method used in correlating the marker combination of the invention e.g. to the absence or presence of CRC is selected from DA (i.e. Linear-, Quadratic-, Regularized Discriminant Analysis), Kernel Methods (i.e. SVM), Nonparametric Methods (i.e. k-Nearest-Neighbor Classifiers), PLS (Partial Least Squares), Tree-Based Methods (i.e. Logic Regression, CART, Random Forest Methods, Boosting Methods), or Generalized Linear Models (i.e. Logistic Regression). Details relating to these statistical methods are found in the following references: Ruczinski, I., et al, J. of Computational and Graphical Statistics, 12 (2003) 475-511; Friedman, J. H. , J. of the American Statistical Association 84 (1989) 165-175; Hastie, Trevor, Tibshirani, Robert, Friedman, Jerome, The Elements of Statistical Learning, Springer Series in Statistics, 2001; Breiman, L., et al., Machine Learning, 45 (2001) 5-32; Pepe, M. S., The Statistical Evaluation of Medical Tests for Classification and Prediction, Oxford Statistical Science Series, 28 (2003); and Duda, R. O., Hart, P. E., Stork, D. G., Pattern Classification, Wiley Interscience, 2nd Edition (2001).

It is a preferred embodiment of the invention to use an optimized multivariate cut-off for the underlying combination of biological markers and to discriminate state A from state B, e.g. diseased from healthy. In this type of analysis the markers are no longer independent but form a marker panel. It could be established that combining the measurements of MDP1, NSE and CYFRA 21-1, does particularly improve the diagnostic accuracy for CRC as compared to either healthy controls or, as also assessed, as compared to healthy controls plus non-malignant disease

controls. Especially the later finding is of great importance, because a patient with a non-malignant disease may require quite a different treatment as a patient with CRC.

5 Accuracy of a test is best described by its receiver-operating characteristics (ROC) (see especially Zweig, M. H., and Campbell, G., Clin. Chem. 39 (1993) 561-577). The ROC graph is a plot of all of the sensitivity/specificity pairs resulting from continuously varying the decision threshold over the entire range of data observed.

10 The clinical performance of a laboratory test depends on its diagnostic accuracy, or the ability to correctly classify subjects into clinically relevant subgroups. Diagnostic accuracy measures the test's ability to correctly distinguish two different conditions of the subjects investigated. Such conditions are for example health and disease or benign versus malignant disease.

15 In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity versus $1 - \text{specificity}$ for the complete range of decision thresholds. On the y-axis is sensitivity, or the true-positive fraction [defined as (number of true-positive test results) / (number of true-positive + number of false-negative test results)]. This has also been referred to as positivity in the presence of a disease or condition. It is calculated solely from the affected subgroup. On the x-axis is the false-positive fraction, or $1 - \text{specificity}$ [defined as (number of false-positive results) / (number of true-negative + number of false-positive results)]. It is an index of specificity and is calculated entirely from the unaffected subgroup. Because the true- and false-positive fractions are calculated entirely separately, by using the test results from two different subgroups, the ROC plot is independent of the prevalence of disease in the sample. Each point on the ROC plot represents a sensitivity/ $1 - \text{specificity}$ pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions of results) has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0, or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity). The theoretical plot for a test with no discrimination (identical distributions of results for the two groups) is a 45° diagonal line from the lower left corner to the upper right corner. Most plots fall in between these two extremes. (If the ROC plot falls completely below the 45° diagonal, this is easily remedied by

reversing the criterion for "positivity" from "greater than" to "less than" or vice versa.) Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the test.

5 One convenient goal to quantify the diagnostic accuracy of a laboratory test is to express its performance by a single number. The most common global measure is the area under the ROC plot. By convention, this area is always ≥ 0.5 (if it is not, one can reverse the decision rule to make it so). Values range between 1.0 (perfect separation of the test values of the two groups) and 0.5 (no apparent distributional difference between the two groups of test values). The area does not depend only on
10 a particular portion of the plot such as the point closest to the diagonal or the sensitivity at 90% specificity, but on the entire plot. This is a quantitative, descriptive expression of how close the ROC plot is to the perfect one (area = 1.0).

Combining measurements of MDP1 with other recently discovered markers, like CYFRA 21-1 or NMMT or with known markers like CEA and NSE, or with other
15 markers of CRC yet to be discovered, leads and will lead, respectively, to further improvements in assessment of CRC.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the
20 procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 The figure shows a 2D-gel, loaded with a tumor sample (left side), and a gel, loaded with a matched control sample (right side) obtained from adjacent healthy mucosa. The apparent molecular weight and the isoelectric point of MDP1 are around 50-60 kDa and 5.5, respectively. The determined molecular weight corresponds well to the theoretical value of 41 kDa of the mature form of the peptide backbone, i.e. after removal of the N-terminal signal peptide and the C-terminal propeptide, to which about 10-20 kDa have to be added due to N-linked glycosylation of the protein. The circle in the enlarged section of these gels indicates
25
30

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the position for the protein MDP1. This protein was not detectable by the same method in healthy mucosa of the same patient.

Figure 2

5 The figure shows a typical example of a Western-Blot. The gel was loaded with tissue lysates from colorectal tumor tissue and adjacent healthy control tissue from 4 patients (subject 19: colon ca (carcinoma), Dukes C; subject 22: colon ca, Dukes B; subject 10 28: colon ca, Dukes B; and subject 34: colon ca, Dukes B). Presence of MDP1 in the samples was tested using a polyclonal rabbit anti-MDP1 serum. Lanes containing tumor lysates are indicated with "T", lanes containing normal control tissue with "N". The marker lane containing a molecular weight protein standard is indicated by "Ma". The lane containing recombinant MDP1 is indicated by "rec Ag". The recombinant antigen, which 15 is not glycosylated since it was expressed in E.coli, has an apparent molecular weight of 41 kDa, well in line with the theoretical size of the polypeptide chain. In contrast, MDP1 from tumor tissue migrates with an apparent molecular with of 50-60 kDa, which is expected due to N-glycosylation of MDP1 and in accordance the findings of the 2D-gel analysis (see Figure 1)

20 In this typical example, 3 of 4 tumor samples give a strong signal at the position of MDP1, whereas no or only a very weak signal can be detected in the lysates from adjacent normal control tissue. These findings indicate a strong overexpression of MDP1 in 25 tumor tissue from colorectal cancer patients.

Abbreviations

30	ABTS	2,2'-Azino-di- [3-ethylbenzthiazoline sulfonate (6)] diammonium salt
	BSA	bovine serum albumin
	cDNA	complementary DNA
	CHAPS	(3-[(3-Cholamidopropyl)-dimethylammonio]- 1-propane-sulfonate)

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	DMSO	dimethyl sulfoxide
	DTT	dithiothreitol
	EDTA	ethylene diamine tetraacetic acid
	ELISA	enzyme-linked immunosorbent assay
5	HRP	horseradish peroxidase
	IAA	iodacetamid
	IgG	immunoglobulin G
	IEF	isoelectric focussing
	IPG	immobilized pH gradient
10	LDS	lithium dodecyl sulfate
	MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
	MES	mesityl, 2,4,6-trimethylphenyl
	OD	optical density
15	PAGE	polyacrylamide gel electrophoresis
	PBS	phosphate buffered saline
	PI	isoelectric point
	RTS	rapid translation system
	SDS	sodium dodecyl sulfate

20

Example 1

Identification of MDP1 as a potential colorectal cancer marker

Sources of tissue

25 In order to identify tumor-specific proteins as potential diagnostic markers for colorectal cancer, analysis of three different kinds of tissue using proteomics methods is performed.

30 Tissue specimen from a patient suffering from colorectal cancer are analyzed. Three different tissue types are collected from a therapeutic resection: tumor tissue (> 80% tumor) (T), adjacent healthy tissue (N) and stripped mucosa from adjacent healthy mucosa (M). The latter two tissue types serve as matched healthy control samples. Tissues are immediately snap frozen after resection and stored at – 80°C before processing. Tumors are diagnosed by histopathological criteria.

Tissue preparation

0.8-1.2 g of frozen tissue are put into a mortar and completely frozen by liquid nitrogen. The tissue is pulverized in the mortar, dissolved in the 10-fold volume (w/v) of lysis buffer (40 mM Na-citrate, 5 mM MgCl₂, 1% Genapol X-080, 0.02% Na-azide, Complete[®] EDTA-free [Roche Diagnostics GmbH, Mannheim, Germany, Cat. No. 1 873 580]) and subsequently homogenized in a Wheaton[®] glass homogenizer (20 x loose fitting, 20 x tight fitting). 3 ml of the homogenate are subjected to a sucrose-density centrifugation (10-60% sucrose) for 1 h at 4500 x g. After this centrifugation step three fractions are obtained. The fraction on top of the gradient contains the soluble proteins and is used for further analysis.

Isoelectric focussing (IEF) and SDS-PAGE

For IEF, 3 ml of the suspension are mixed with 12 ml sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.4% IPG buffer pH 4-7, 0.5% DTT) and incubated for 1 h. The samples are concentrated in an Amicon[®] Ultra-15 device (Millipore GmbH, Schwalbach, Germany) and the protein concentration is determined using the Bio-Rad[®] protein assay (Cat.No. 500-0006; Bio-Rad Laboratories GmbH, München, Germany) following the instructions of the supplier's manual. To a volume corresponding to 1.5 mg of protein sample buffer is added to a final volume of 350 µl. This solution is used to rehydrate IPG strips pH 4-7 (Amersham Biosciences, Freiburg, Germany) overnight. The IEF is performed using the following gradient protocol: 1.) 1 minute to 500 V; 2.) 2 h to 3,500 V; 3.) 22 h at constant 3,500 V giving rise to 82 kVh. After IEF, strips are stored at -80°C or directly used for SDS-PAGE.

Prior to SDS-PAGE the strips are incubated in equilibration buffer (6 M urea, 50 mM Tris/HCl, pH 8.8, 30% glycerol, 2% SDS), for reduction DDT (15 min, + 50 mg DTT/10 ml), and for alkylation IAA (15 min, + 235 mg iodacetamide/10 ml) is added. The strips are put on 12.5% polyacrylamide gels and subjected to electrophoresis at 1 W/gel for 1 h and thereafter at 17 W/gel. Subsequently, the gels are fixed (50% methanol, 10% acetate) and stained overnight with Novex[™] Colloidal Blue Staining Kit (Invitrogen, Karlsruhe, Germany, Cat No. LC6025, 45-7101).

Detection of MDP1 as a potential marker for colorectal cancer

Tumor and control samples are analyzed as follows. All spots of each gel are excised by a picking robot and the proteins present in the spots are identified by MALDI-TOF mass spectrometry (Ultraflex™ Tof/Tof, Bruker Daltonik GmbH, Bremen, Germany). For a given patient, 4 gels from the tumor sample are compared with 4 gels each from adjacent normal and stripped mucosa tissue and analyzed for distinctive spots corresponding to differentially expressed proteins. By this means, protein MDP1 is found to be specifically expressed in tumor tissue and not detectable in healthy control tissue of a patient. It therefore – amongst many other proteins - qualifies as a candidate marker for use in the diagnosis of colorectal cancer.

Example 2

Generation of antibodies to the colorectal cancer marker protein MDP1

Polyclonal antibody to the colorectal cancer marker protein MDP1 is generated for further use of the antibody in the measurement of serum and plasma and blood levels of MDP1 by immunodetection assays, e.g. Western Blotting and ELISA.

Recombinant protein expression in E. coli

In order to generate antibodies to MDP1, recombinant expression of the protein is performed for obtaining immunogens. The expression is done applying a combination of the RTS 100 expression system and E.coli. In a first step, the DNA sequence is analyzed and recommendations for high yield cDNA silent mutational variants and respective PCR-primer sequences are obtained using the “ProteoExpert RTS E.coli HY” system. This is a commercial web based service (www.proteoexpert.com). Using the recommended primer pairs, the “RTS 100 E. coli Linear Template Generation Set, His-tag” (Roche Diagnostics GmbH, Mannheim, Germany, Cat.No. 3186237) system to generate linear PCR templates from the cDNA and for in-vitro transcription and expression of the nucleotide sequence coding for the MDP1 protein is used. For Western-blot detection and later purification, the expressed protein contains a His-tag. The best expressing variant is identified. All steps from PCR to expression and detection are carried out according to the instructions of the manufacturer. The respective PCR product,

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containing all necessary T7 regulatory regions (promoter, ribosomal binding site and T7 terminator) is cloned into the pBAD TOPO[®] vector (Invitrogen, Karlsruhe, Germany, Cat. No. K 4300/01) following the manufacturer's instructions. For expression using the T7 regulatory sequences, the construct is transformed into E. coli BL 21 (DE 3) (Studier, F.W., et al., Methods Enzymol. 185 (1990) 60-89) and the transformed bacteria are cultivated in a 1 l batch for protein expression.

Purification of His-MDP1 fusion protein is done following standard procedures on a Ni-chelate column. Briefly, 1 l of bacteria culture containing the expression vector for the His-MDP1 fusion protein is pelleted by centrifugation. The cell pellet is resuspended in lysis buffer, containing phosphate, pH 8.0, 7 M guanidium chloride, imidazole and thioglycerole, followed by homogenization using a Ultra-Turrax[®]. Insoluble material is pelleted by high speed centrifugation and the supernatant is applied to a Ni-chelate chromatographic column. The column is washed with several bed volumes of lysis buffer followed by washes with buffer, containing phosphate, pH 8.0 and Urea. Finally, bound antigen is eluted using a phosphate buffer containing SDS under acid conditions.

Production of monoclonal antibodies against the MDP1

a) Immunization of mice

12 week old A/J mice are initially immunized intraperitoneally with 100 µg MDP1. This is followed after 6 weeks by two further intraperitoneal immunizations at monthly intervals. In this process each mouse is administered 100 µg MDP1 adsorbed to aluminum hydroxide and 10⁹ germs of *Bordetella pertussis*. Subsequently the last two immunizations are carried out intravenously on the 3rd and 2nd day before fusion using 100 µg MDP1 in PBS buffer for each.

b) Fusion and cloning

Spleen cells of the mice immunized according to a) are fused with myeloma cells according to Galfre, G., and Milstein, C., Methods in Enzymology 73 (1981) 3-46. In this process ca. 1*10⁸ spleen cells of the immunized mouse are mixed with 2x10⁷ myeloma cells (P3X63-Ag8-653, ATCC CRL1580) and centrifuged (10 min at 300 x g and 4°C.). The cells are then washed once with RPMI 1640 medium without

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fetal calf serum (FCS) and centrifuged again at 400 x g in a 50 ml conical tube. The supernatant is discarded, the cell sediment is gently loosened by tapping, 1 ml PEG (molecular weight 4000, Merck, Darmstadt) is added and mixed by pipetting. After 1 min in a water-bath at 37°C., 5 ml RPMI 1640 without FCS is added drop-wise at room temperature within a period of 4-5 min. Afterwards 5 ml RPMI 1640 containing 10% FCS is added drop-wise within ca. 1 min, mixed thoroughly, filled to 50 ml with medium (RPMI 1640+10% FCS) and subsequently centrifuged for 10 min at 400 x g and 4°C. The sedimented cells are taken up in RPMI 1640 medium containing 10% FCS and sown in hypoxanthine-azaserine selection medium (100 mmol/l hypoxanthine, 1 µg/ml azaserine in RPMI 1640+10% FCS). Interleukin 6 at 100 U/ml is added to the medium as a growth factor.

After ca. 10 days the primary cultures are tested for specific antibody. MDP1-positive primary cultures are cloned in 96-well cell culture plates by means of a fluorescence activated cell sorter. In this process again interleukin 6 at 100 U/ml is added to the medium as a growth additive.

c) Immunoglobulin isolation from the cell culture supernatants

The hybridoma cells obtained are sown at a density of 1×10^5 cells per ml in RPMI 1640 medium containing 10% FCS and proliferated for 7 days in a fermenter (Thermodux Co., Wertheim/Main, Model MCS-104XL, Order No. 144-050). On average concentrations of 100 µg monoclonal antibody per ml are obtained in the culture supernatant. Purification of this antibody from the culture supernatant is carried out by conventional methods in protein chemistry (e.g. according to Bruck, C., et al., Methods in Enzymology 121 (1986) 587-695).

Generation of polyclonal antibodies

25 a) Immunization

For immunization, a fresh emulsion of the protein solution (100 µg/ml protein MDP1) and complete Freund's adjuvant at the ratio of 1:1 is prepared. Each rabbit is immunized with 1 ml of the emulsion at days 1, 7, 14 and 30, 60 and 90. Blood is drawn and resulting anti-MDP1 serum used for further experiments as described in examples 3 and 4.

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b) Purification of IgG (immunoglobulin G) from rabbit serum by sequential precipitation with caprylic acid and ammonium sulfate

One volume of rabbit serum is diluted with 4 volumes of acetate buffer (60 mM, pH 4.0). The pH is adjusted to 4.5 with 2 M Tris-base. Caprylic acid (25 µl/ml of diluted sample) is added drop-wise under vigorous stirring. After 30 min the sample is centrifuged (13,000 x g, 30 min, 4°C), the pellet discarded and the supernatant collected. The pH of the supernatant is adjusted to 7.5 by the addition of 2 M Tris-base and filtered (0.2 µm).

The immunoglobulin in the supernatant is precipitated under vigorous stirring by the drop-wise addition of a 4 M ammonium sulfate solution to a final concentration of 2 M. The precipitated immunoglobulins are collected by centrifugation (8,000 x g, 15 min, 4°C).

The supernatant is discarded. The pellet is dissolved in 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl and exhaustively dialyzed. The dialysate is centrifuged (13,000 x g, 15 min, 4°C) and filtered (0.2 µm).

Biotinylation of polyclonal rabbit IgG

Polyclonal rabbit IgG is brought to 10 mg/ml in 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl. Per ml IgG solution 50 µl Biotin -N-hydroxysuccinimide (3.6 mg/ml in DMSO) are added. After 30 min at room temperature, the sample is chromatographed on Superdex 200 (10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl). The fraction containing biotinylated IgG are collected. Monoclonal antibodies are biotinylated according to the same procedure.

Digoxigenylation of polyclonal rabbit IgG

Polyclonal rabbit IgG is brought to 10 mg/ml in 10 mM NaH₂PO₄/NaOH, 30 mM NaCl, pH 7.5. Per ml IgG solution 50 µl digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Roche Diagnostics, Mannheim, Germany, Cat. No. 1 333 054) (3.8 mg/ml in DMSO) are added. After 30 min at room temperature, the sample is chromatographed on Superdex® 200 (10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl). The fractions containing digoxigenylated

IgG are collected. Monoclonal antibodies are labeled with digoxigenin according to the same procedure.

Example 3

5 Western Blotting for the detection of MDP1 in human colorectal cancer tissue using polyclonal antibody as generated in Example 2.

Tissue lysates from tumor samples and healthy control samples are prepared as described in Example 1, "Tissue preparation".

10 SDS-PAGE and Western-Blotting are carried out using reagents and equipment of Invitrogen, Karlsruhe, Germany. For each tissue sample tested, 10 µg of tissue lysate are diluted in reducing NuPAGE® (Invitrogen) SDS sample buffer and heated for 10 min at 95°C. Samples are run on 4-12% NuPAGE® gels (Tris-Glycine) in the MES running buffer system. The gel-separated protein mixture is blotted onto nitrocellulose membranes using the Invitrogen XCell II™ Blot Module (Invitrogen) and the NuPAGE® transfer buffer system. The membranes are washed 3 times in 15 PBS/0.05% Tween-20 and blocked with Roti®-Block blocking buffer (A151.1; Carl Roth GmbH, Karlsruhe, Germany) for 2 h. The primary antibody, polyclonal rabbit anti-MDP1 serum (generation described in Example 2), is diluted 1:10,000 in Roti®-Block blocking buffer and incubated with the membrane for 1 h. The 20 membranes are washed 6 times in PBS/0.05% Tween-20. The specifically bound primary rabbit antibody is labeled with a POD-conjugated polyclonal sheep anti-rabbit IgG antibody, diluted to 10 mU/ml in 0.5 x Roti®-Block blocking buffer. After incubation for 1 h, the membranes are washed 6 times in PBS/0.05% Tween-20. For detection of the bound POD-conjugated anti-rabbit antibody, the 25 membrane is incubated with the Lumi-Light^{PLUS} Western Blotting Substrate (Order-No. 2015196, Roche Diagnostics GmbH, Mannheim, Germany) and exposed to an autoradiographic film.

Results of a typical experiment are shown in Figure 2. The strong overexpression of MDP1 in tumor tissue versus adjacent control tissue is found in 11 out of 14 subjects with colorectal cancer tested.

Example 4

ELISA for the measurement of MDP1 in human serum and plasma samples.

For detection of MDP1 in human serum or plasma, a sandwich ELISA is developed. For capture and detection of the antigen, aliquots of the anti-MDP1 polyclonal antibody (see Example 2) are conjugated with biotin and digoxigenin, respectively.

Streptavidin-coated 96-well microtiter plates are incubated with 100 µl biotinylated anti-MDP1 polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween-20. After incubation, plates are washed three times with 0.9% NaCl, 0.1% Tween-20. Wells are then incubated for 2 h with either a serial dilution of the recombinant protein (see Example 2) as standard antigen or with diluted plasma samples from patients. After binding of MDP1, plates are washed three times with 0.9% NaCl, 0.1% Tween-20. For specific detection of bound MDP1, wells are incubated with 100 µl of digoxigenylated anti-MDP1 polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween-20. Thereafter, plates are washed three times to remove unbound antibody. In a next step, wells are incubated with 20 mU/ml anti-digoxigenin-POD conjugates (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1633716) for 60 min in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween-20. Plates are subsequently washed three times with the same buffer. For detection of antigen-antibody complexes, wells are incubated with 100 µl ABTS solution (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 11685767) and OD is measured after 30-60 min at 405 nm with an ELISA reader.

Example 5

ROC analysis to assess clinical utility in terms of diagnostic accuracy.

Accuracy is assessed by analyzing individual liquid samples obtained from well-characterized patient cohorts, i.e., 50 patients having undergone colonoscopy and found to be free of adenoma or CRC, 50 patients diagnosed and staged as T1-3, N0, M0 of CRC, and 50 patients diagnosed with progressed CRC, having at least tumor infiltration in at least one proximal lymph node or more severe forms of metastasis, respectively. CEA as measured by a commercially available assay (Roche Diagnostics, CEA-assay (Cat. No. 1 173 1629 for Elecsys® Systems immunoassay

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analyzer) and MDP1 measured as described above are quantified in a serum obtained from each of these individuals. ROC-analysis is performed according to Zweig, M. H., and Campbell, *supra*. Discriminatory power for differentiating patients in the group T_{is}-3, N0, M0 from healthy individuals for the combination of MDP1 with the established marker CEA is calculated by regularized discriminant analysis (Friedman, J. H., Regularized Discriminant Analysis, Journal of the American Statistical Association 84 (1989) 165-175).

Preliminary data indicate that MDP1 may also be very helpful in the follow-up of patients after surgery.

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WO 2004/057336

Zweig, M. H., and Campbell, G., Clin. Chem. 39 (1993) 561-577

Patent Claims

1. A method for assessing colorectal cancer *in vitro* comprising the steps of
 - a) measuring in a sample the concentration of MDP1 (= dehydropeptidase-I),
 - 5 b) optionally measuring in a sample the concentration of one or more other marker of colorectal cancer, and
 - c) correlating the concentration determined in step (a) and optionally the concentration(s) determined in step (b) to the diagnosis of colorectal cancer.
- 10 2. The method according to claim 1, further characterized in that said sample is serum.
3. The method according to claim 1, further characterized in that said sample is plasma.
- 15 4. The method according to claim 1, further characterized in that said sample is whole blood.
5. The method according to any of the claims 1 to 4, further characterized in that said one or more other marker is selected from the group consisting of NSE, CYFRA 21-1, NNMT, CA 19-9, CA 72-4, and CEA.
- 20 6. Use of protein MDP1 as a marker molecule in the assessment of colorectal cancer.
7. Use of a marker panel comprising MDP1 and one or more other marker for colorectal cancer in the assessment of colorectal cancer
8. Use of the marker panel according to claim 7, wherein the one or more other marker is selected from the group consisting of NSE, CYFRA 21-1, NNMT, 25 CA 19-9, CA 72-4, and CEA..
9. A kit for performing the method according to claim 1 comprising the reagents required to measure MDP1.

- 40 -

10. A kit for performing the method according to claim 5 comprising the reagents required to measure MDP1 and one or more other marker of colorectal cancer.

Figure 1:

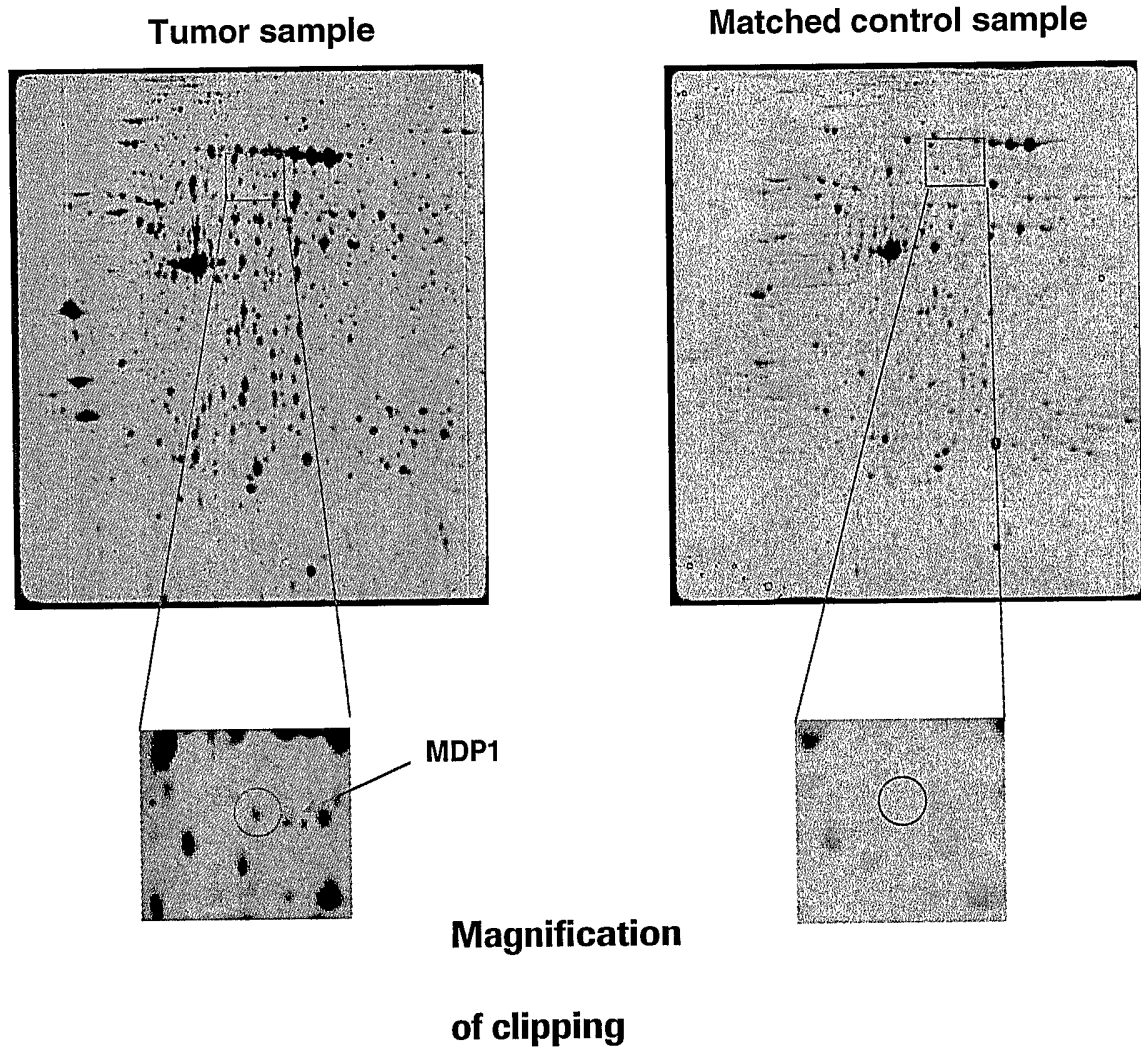
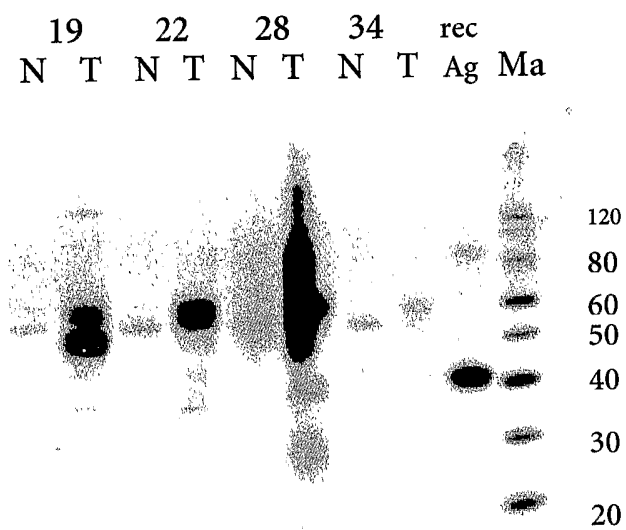


Figure 2:

- 1 -

SEQUENCE LISTING

<110> Roche Diagnostics GmbH
 F. Hoffmann-La Roche AG

<120> Use of protein MDP1 as a marker for colorectal cancer

<130> 22893

<150> EP 04030620.1
 <151> 2004-12-23

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 (Dehydropeptidase-I) (Renal dipeptidase) (RDP) - Homo sapiens
 (Human).

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/013868

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/574 G01N33/573

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

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

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/022863 A (THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE; BUCKHAULTS, PHILLIP;) 20 March 2003 (2003-03-20)	1-4,6,9
Y	paragraphs [0027], [0030], [0031]; claims 11,15,49,50	1-10
X	MCIVER C M ET AL: "Dipeptidase 1: a candidate tumor-specific molecular marker in colorectal carcinoma" CANCER LETTERS, vol. 209, no. 1, 8 June 2004 (2004-06-08), pages 67-74, XP002368418 ISSN: 0304-3835 cited in the application	1-4,6,9
Y	abstract paragraphs [03.1], [03.2]; figure 1	1-10

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

2 March 2006

Date of mailing of the international search report

20/03/2006

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2005/013868

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KOJIMA O ET AL: "[Clinical significance of serum neuron specific enolase (NSE) levels in patients with gastric and colorectal cancer]" RINSHO BYORI. THE JAPANESE JOURNAL OF CLINICAL PATHOLOGY. NOV 1986, vol. 34, no. 11, November 1986 (1986-11), pages 1236-1240, XP008047371 ISSN: 0047-1860 the whole document	5,7,8,10
Y	ZUO QIANG ET AL: "Diagnostic value of multi-tumor markers protein biochip detective system for digestive tract cancer" ZHONGGUO ZHONGLIU LINCHUANG, vol. 31, no. 6, March 2004 (2004-03), pages 337-339, XP008047370 ISSN: 1000-8179 abstract	5,7,8,10
Y	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; February 2000 (2000-02), MIYASHITA T ET AL: "Evaluation of combination assay of serum CEA, CYFRA 21-1, IAP and CA 19-9 in colorectal cancer" XP002329220 Database accession no. PREV200000195119 abstract & MIYASHITA T ET AL: "Evaluation of combination assay of serum CEA, CYFRA21-1, IAP and CA19-9 in colorectal cancer" JOURNAL OF THE JAPAN SOCIETY OF COLOPROCTOLOGY, vol. 53, no. 2, February 2000 (2000-02), pages 76-82, XP008047338 ISSN: 0047-1801 abstract	5,7,8,10
Y	HOFMANN D ET AL: "The prognostic value of CEA, CA242, CA19-9, CA72-4, CYFRA 21-1 and S100 in colorectal cancer." TUMOR BIOLOGY, vol. 24, no. Supplement 1, August 2003 (2003-08), page 49, XP008047335 & 31ST MEETING OF THE INTERNATIONAL SOCIETY FOR ONCODEVELOPMENTAL BIOLOGY AND MEDICINE; EDINBURGH, UK; AUGUST 30-SEPTEMBER 04, 2003 ISSN: 1010-4283 abstract	5,7,8,10

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2005/013868

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; March 2002 (2002-03), MATSUMOTO H: "Clinical significance of the mesenteric blood level of CYFRA21-1 in patients with colorectal cancer: Patients at risk for postoperative hematogenous metastasis" XP002329221 Database accession no. PREV200200296087 abstract & MATSUMOTO H: "Clinical significance of the mesenteric blood level of CYFRA21-1 in patients with colorectal cancer: Patients at risk for postoperative hematogenous metastasis" JOURNAL OF THE JAPAN SOCIETY OF COLOPROCTOLOGY, vol. 55, no. 3, March 2002 (2002-03), pages 136-144, XP008047337 ISSN: 0047-1801 abstract	5,7,8,10
Y	----- SAKAHARA H ET AL: "Evaluation of a cytokeratin 19 assay kit 'BALL ELSA CYFRA21-1'" KAKUIGAKU 1993 JAPAN, vol. 30, no. 12, 1993, pages 1475-1479, XP008047336 ISSN: 0022-7854 page 1479	5,8,10
Y	----- WO 2004/057336 A (ROCHE DIAGNOSTICS GMBH; F. HOFFMANN-LA ROCHE AG; BERNDT, PETER; HAGMAN) 8 July 2004 (2004-07-08) cited in the application abstract -----	5,8,10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2005/013868

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 03022863	A	20-03-2003	EP	1430071 A1		23-06-2004
			JP	2005518781 T		30-06-2005
WO 2004057336	A	08-07-2004	AU	2003296684 A1		14-07-2004
			BR	0317394 A		16-11-2005
			CA	2510377 A1		08-07-2004
			US	2006003368 A1		05-01-2006