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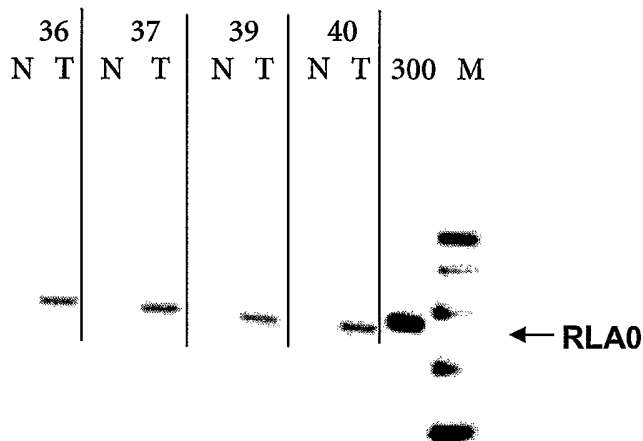
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(54) Title: USE OF PROTEIN ACIDIC RIBOSOMAL PROTEIN P0 (RLA-0) AS A MARKER FOR COLORECTAL CANCER



(57) Abstract: The present invention relates to the diagnosis of colorectal cancer. It discloses the use of protein RLA-0 (60S acidic ribosomal protein P0) in the diagnosis of colorectal cancer. It relates to a method for diagnosis of colorectal cancer from a stool sample, derived from an individual by measuring RLA-0 in said sample. Measurement of RLA-0 can, e.g., be used in the early detection or diagnosis of colorectal cancer.

USE OF PROTEIN ACIDIC RIBOSOMAL PROTEIN P0 (RLA-0) AS A MARKER FOR COLORECTAL CANCER

The present invention relates to the diagnosis of colorectal cancer. It discloses the use of the protein 60S acidic ribosomal protein P0 (= RLA-0) in the diagnosis of colorectal cancer. Furthermore, it especially relates to a method for diagnosis of
5 colorectal cancer from a stool sample, derived from an individual by measuring RLA-0 in said sample. Measurement of RLA-0 can, e.g., be used in the early detection or diagnosis of colorectal cancer.

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
10 the most frequent cancers in the Western world.

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.
15 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.

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

The earliest detection procedures available at present for colorectal cancer involve
20 using tests for fecal blood or endoscopic procedures. However, significant tumor size must typically exist before fecal blood is detected. With regard to detection of CRC from a stool sample, the current state of the art is the guaiac-based fecal occult blood test.

In the recent years a tremendous amount of so-called colon specific or even so-called colorectal cancer specific genes has been reported. The vast majority of the
25 corresponding research papers or patent applications are based on data obtained by analysis of RNA expression patterns in colon (cancer) tissue versus a different tissue or an adjacent normal tissue, respectively. Such approaches may be summarized as differential mRNA display techniques.

As an example for data available from mRNA-display techniques, WO 01/96390 shall be mentioned and discussed. This application describes and claims more than two hundred isolated polynucleotides and the corresponding polypeptides as such, as well as their use in the detection of CRC. However, it is general knowledge that
5 differences on the level of mRNA are not mirrored by the level of the corresponding proteins. A protein encoded by a rare mRNA may be found in very high amounts and a protein encoded by an abundant mRNA may nonetheless be hard to detect and find at all. This lack of correlation between mRNA-level and protein level is due to reasons like mRNA stability, efficiency of translation, stability of the protein,
10 etc.

There also are recent approaches investigating the differences in protein patterns between different tissues or between healthy and diseased tissue in order to identify candidate marker molecules which might be used in the diagnosis of CRC. Brünagel, G., et al., Cancer Research 62 (2002) 2437-2442, have identified seven
15 nuclear matrix proteins which appear to be more abundant in CRC tissue as compared to adjacent normal tissue. No data from liquid and stool samples obtained from an individual are reported.

WO 02/078636 reports about nine colorectal cancer-associated spots as found by surface-enhanced laser desorption and ionization (SELDI). These spots are seen
20 more frequently in sera obtained from patients with CRC as compared to sera obtained from healthy controls. However, the identity of the molecule(s) comprised in such spot, e.g., its (their sequence), is not known.

Despite the large and ever growing list of candidate protein markers in the field of CRC, to date clinical/diagnostic utility of these molecules is not known. In order to
25 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
30 characteristics, which will be described in detail below.

At present, for example, 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
5 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
10 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).

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
15 cancer in the asymptomatic population (Reynoso, G., et al., JAMA 220 (1972) 361-365; Sturgeon, C., Clinical Chemistry 48 (2002) 1151-1159).

Samples taken from stool have the advantage that such sampling is easily possible by non-invasive means.

As mentioned above, the guaiac test is currently most widely used as a screening
20 assay for CRC from stool. The guaiac test, however, has both poor sensitivity as well as poor specificity. 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
25 detection, but colonoscopy is invasive with significant costs, risks, and complications (Silvis, S.E., et al., JAMA 235 (1976) 928-930; 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).

The sensitivity and specificity of diagnostic alternatives to the guaiac test have been
30 recently investigated by Sieg, A., et al., Int. J. Colorectal Dis. 14 (1999) 267-271. Especially the measurement of hemoglobin and of the hemoglobin-haptoglobin complex from stool specimen have been compared. It has been noted that the

hemoglobin assay has an unsatisfactory sensitivity for the detection of colorectal neoplasms. Whereas cancer in its progressed carcinoma stage is detected with a sensitivity of about 87% the earlier tumor stages are not detected with a sufficient sensitivity. The hemoglobin-haptoglobin complex assay was more sensitive in the
5 detection of earlier stages of CRC. This more sensitive detection was accompanied by a poor specificity. Since poor specificity, however, translates to a high number of unnecessary secondary investigations, like colonoscopy, an assay with a poor accuracy also does not meet the requirements of a generally accepted screening assay.

- 10 A further alternative method to the guaiac test for detection of CRC in stool has been published recently and consists in the detection of the colorectal cancer-specific antigen, "minichromosome maintenance protein 2" (MCM2) by immunohistochemistry in colonic cells shed into stool. Due to the small study size, conclusion on the diagnostic value for detection of colorectal cancer is preliminary.
15 However, the test seems to have only limited sensitivity to detect right-sided colon cancer. (Davies, R.J., et al., Lancet 359 (2002) 1917-1919).

The identification of an early CRC tumor marker that would allow reliable cancer detection or provide early prognostic information by non-invasive means from a stool specimen could lead to a diagnostic assay that would greatly aid in the
20 diagnosis and in the management of this disease. Therefore, an urgent clinical need exists to improve the diagnosis of CRC, especially from stool. 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.

- 25 It was the task of the present invention to investigate whether a new marker can be identified which may aid in CRC diagnosis. Preferably such marker would be present in stool and allow for a non-invasive diagnosis.

Surprisingly, it has been found that use of protein RLA-0 can at least partially overcome the problems known from the state of the art.

- 30 The present invention therefore relates to a method for the diagnosis of colorectal cancer comprising the steps of a) providing a stool sample obtained from an

individual, b) contacting said sample with a specific binding agent for RLA-0 under conditions appropriate for formation of a complex between said binding agent and RLA-0, and c) correlating the amount of complex formed in (b) to the diagnosis of colorectal cancer.

- 5 In a preferred embodiment the stool sample is processed to obtain a processed sample liquid which is more convenient to handle than a stool specimen. Such processed sample is then incubated with the specific binding agent for RLA-0. The present invention therefore also relates to a method for the diagnosis of colorectal cancer comprising the steps of a) providing a stool sample obtained from an individual, b) processing said sample to obtain a processed liquid sample, c) 10 contacting said processed liquid sample with a specific binding agent for RLA-0 under conditions appropriate for formation of a complex between said binding agent and RLA-0, and d) correlating the amount of complex formed in (c) to the diagnosis of colorectal cancer. A preferred method uses a stool sample obtained 15 from an individual.

- Another preferred embodiment of the invention is a method for the diagnosis of colorectal cancer comprising the steps of a) processing a stool sample obtained from an individual to obtain a processed liquid sample b) contacting said processed liquid sample with a specific binding agent for RLA-0 under conditions appropriate 20 for formation of a complex between said binding agent and RLA-0, and c) correlating the amount of complex formed in (b) to the diagnosis of colorectal cancer.

- In another preferred embodiment the stool sample is processed to retrieve colonocytes which are then smeared on a microscopic slide. Such processed sample 25 is then incubated with the specific binding agent for RLA-0. The present invention therefore also relates to a method for the diagnosis of colorectal cancer comprising the steps of a) providing a stool sample obtained from an individual, b) processing said sample to retrieve colonocytes, c) contacting said processed sample with a specific binding agent for RLA-0 under conditions appropriate for formation of a 30 complex between said binding agent and RLA-0, and d) correlating the amount of complex formed in (c) to the diagnosis of colorectal cancer.

Ribosomes, the organelles that catalyze protein synthesis, consist of a small 40S subunit and a large 60S subunit. Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins (Uechi, T., et al., Genomics (2001) 223-230). RLA0 (60S acidic ribosomal protein P0; SWISS-PROT: P05388) is a 34 kDa-component of the 60S subunit and belongs, together with P1 and P2, to the family of acidic ribosomal phosphoproteins, called P-proteins (Tchorzewski, M., Int. J. Biochem. Cell. Biol. 34 (2002) 911-915). The isolation and analysis of the cDNA coding for the human P-proteins was described by Rich, B.E., and Steitz, J.A., Mol. Cell. Biol. 7 (1987) 4065-4074. P0 interacts with two P1 and two P2 molecules to build a pentameric complex (Gonzalo, P., et al., J. Biol. Chem. 276 (2001) 19762-19769). Different transcript variants were identified, encoding an identical protein (SEQ ID NO: 1).

Under normal growth conditions, ribosomal proteins are synthesized stoichiometrically to produce equimolar supply of ribosomal components (Mager, W.H., Biochim. Biophys. Acta 949 (1988) 1-15). However, regulation of transcriptional activity of the ribosomal protein genes is reported to be less regulated than expected (Bortoluzzi, S., et al., Bioinformatics 17 (2001) 1152-1157). In addition, recent studies provide growing evidence, that ribosomal proteins can also function in various cellular processes such as replication, transcription, DNA repair, and inflammation, independent of their involvement in protein biosynthesis (Wool, I.G., Trends Biochem. Sci. 21 (1996) 164-165; Yamamoto, T., Pathol. Int. 50 (2000) 863-871).

Several ribosomal proteins, not including RLA0 (P0), were identified to be overexpressed in neoplastic tissue as compared to healthy control tissue, e. g. in esophageal (Wang, Q., et al., Gene 263 (2001) 205-209) or prostate cancer (Vaarala, M.H., et al., Int. J. Cancer 78 (1998) 27-32). For RLA0 (P0) it is reported that it was found to be overexpressed in colorectal cancer and colorectal polyps (Pogue-Geile, K., et al., Mol. Cell. Biol. 11 (1991) 3842-3849). Additionally, expression of RLA0 (P0) is increased in hepatocellular carcinoma (Kondoh, N., et al., Cancer Res. 59 (1999) 4990-4996) and colon carcinoma (Barnard, G.F., et al., Cancer Res. 52 (1992) 3067-3072) as assessed by Northern-Blot hybridization. Moreover, in colorectal carcinoma expression of RLA0 (P0) was increasing with increasing Dukes' stage (Barnard, G.F., et al., Cancer Res. 52 (1992) 3067-3072). However, all of these studies were based on analyses carried out on the transcriptional level.

Most recently, Kasai, H., et al., J. Histochem. Cytochem. 51 (2003) 567-574 performed studies on the translational level for 12 ribosomal proteins using immunohistochemistry. They found, that expression levels of two ribosomal proteins (L7 and S11) were significantly enhanced in colorectal cancer cells. The
5 expression of RLA0 (P0) was, however, not assessed in this study.

As obvious to the skilled artisan, the present invention shall not be construed to be limited to the full-length protein RLA-0 of SEQ ID NO:1. Physiological or artificial fragments of RLA-0, secondary modifications of RLA-0, as well as allelic variants of RLA-0 are also encompassed by the present invention. Artificial fragments
10 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 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
15 at least two epitopes of interest appropriate for setting up a sandwich immunoassay.

In preferred embodiments, the novel marker RLA-0 may be used for monitoring as well as for screening purposes. Its use for screening purposes is most preferred.

When used in patient monitoring the diagnostic method according to the present invention may help to assess tumor load, efficacy of treatment and tumor
20 recurrence in the follow-up of patients. Increased levels of RLA-0 are directly correlated to tumor burden. After chemotherapy a short term (few hours to 14 days) increase in RLA-0 may serve as an indicator of tumor cell death. In the follow-up of patients (from 3 months to 10 years) an increase of RLA-0 can be used as an indicator for tumor recurrence in the colorectum.

25 In a preferred embodiment the diagnostic method according to the present invention is used for screening purposes. I.e., it is used to assess subjects without a prior diagnosis of CRC by measuring the level of RLA-0 in a stool sample and correlating the level measured to the presence or absence of CRC.

Colorectal cancer most frequently progresses from adenomas (polyps) to malignant
30 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
5 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), Sobin, L.H., Wittekind, Ch. (eds): TNM Classification of Malignant Tumours, fifth
10 edition, 1997.

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,
15 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.

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
20 (neither proximal nor distal), i.e., adenoma, T_{is}, N0, M0 or T1-4; N0; M0 are present. T_{is} denotes carcinoma *in situ*.

In a preferred embodiment the detection of RLA-0 is used to diagnose CRC as early as in the adenoma stage.

It is further preferred, that CRC is diagnosed when it has not yet fully grown
25 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).

The diagnostic method according to the present invention is based on a stool sample which is derived from an individual. The stool sample is extracted and

RLA-0 is specifically measured from this processed stool sample by use of a specific binding agent.

A specific binding agent is, e.g., a receptor for RLA-0, a lectin binding to RLA-0 or an antibody to RLA-0. A specific binding agent has at least an affinity of 10^7 l/mol
5 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 biomolecules present in the sample do not significantly bind to with the binding agent specific for RLA-0. Preferably, the level of binding to a biomolecule other
10 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 specificity.

A specific binding agent preferably is an antibody reactive with RLA-0. The term
15 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.

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
20 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 specific isolation of antibodies. By these means the quality of polyclonal antibodies and hence their performance in immunoassays can be enhanced. (Tijssen, P., *supra*,
25 pages 108-115).

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
30 with constant properties, they represent ideal tools in development of an assay for clinical routine. The generation and use of monoclonal antibodies to RLA-0 in a method according to the present invention is yet another preferred embodiment.

As the skilled artisan will appreciate now, that RLA-0 has been identified as a marker which is useful in the diagnosis of CRC, alternative ways may be used to reach a result comparable to the achievements of the present invention. For example, alternative strategies to generate antibodies may be used. Such strategies
5 comprise amongst others the use of synthetic peptides, representing an epitope of RLA-0 for immunization. Alternatively, DNA Immunization also known as DNA vaccination may be used.

For measurement, the stool sample is obtained from an individual. An aliquot of the stool sample may be used directly. Preferably an aliquot of the stool sample is
10 processed to yield a liquid sample.

The stool sample is preferably used or processed directly after sampling or stored cooled or more conveniently stored frozen. Frozen stool samples can be processed by thawing, followed by dilution in an appropriate buffer, mixing and centrifugation. Supernatants are used as liquid sample for subsequent measurement
15 of marker RLA-0.

An aliquot of the processed stool sample is incubated with the specific binding agent for RLA-0 under conditions appropriate for formation of a binding agent RLA-0-complex. Such conditions need not be specified, since the skilled artisan without any inventive effort can easily identify such appropriate incubation
20 conditions.

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 specific binding agent RLA-0-complex all described in detail in relevant
25 textbooks (cf., e.g., Tijssen P., *supra*, or Diamandis, E.P., et al., eds. (1996) Immunoassay, Academic Press, Boston).

Preferably RLA-0 is detected in a sandwich type assay format. In such assay a first specific binding agent is used to capture RLA-0 on the one side and a second specific binding agent, which is labeled to be directly or indirectly detectable is used
30 on the other side.

As mentioned above, it has surprisingly been found that RLA-0 can be measured from a stool sample obtained from an individual sample. No tissue and no biopsy sample is required to apply the marker RLA-0 in the diagnosis of CRC.

Whereas application of routine proteomics methods to tissue samples, leads to the
5 identification of many potential marker candidates for the tissue selected, the inventors of the present invention have surprisingly been able to detect protein RLA-0 in a stool sample. Even more surprising they have been able to demonstrate that the presence of RLA-0 in such stool sample obtained from an individual can be correlated to the diagnosis of colorectal cancer.

10 Antibodies to RLA-0 with great advantage can also be used in established procedures, e.g., to detect colorectal cancer cells in situ, in biopsies, or in immunohistological procedures.

Preferably, an antibody to RLA-0 is used in a qualitative (RLA-0 present or absent) or quantitative (RLA-0 amount is determined) immunoassay.

15 Measuring the level of protein RLA-0 has proven very advantageous in the field of CRC. Therefore, in a further preferred embodiment, the present invention relates to use of protein RLA-0 as a marker molecule in the diagnosis of colorectal cancer from a stool sample obtained from an individual.

The term marker molecule is used to indicate that an increased level of the analyte
20 RLA-0 as measured from a bodily fluid or especially a processed stool sample obtained from an individual marks the presence of CRC.

It is especially preferred to use the novel marker RLA-0 in the early diagnosis of colorectal cancer.

The use of protein RLA-0 itself, represents a significant progress to the challenging
25 field of CRC diagnosis from stool. Combining measurements of RLA-0 with other known markers, like hemoglobin or the hemoglobin-haptoglobin complex, or with other markers of CRC yet to be discovered, leads to further improvements. Therefore in a further preferred embodiment the present invention relates to the use of RLA-0 as a marker molecule for colorectal cancer in combination with one

or more other marker molecules for colorectal cancer in the diagnosis of colorectal cancer from a stool sample obtained from an individual. Preferred selected other CRC markers with which the measurement of RLA-0 may be combined are hemoglobin and/or the hemoglobin-haptoglobin complex. Very preferred the marker RLA-0 is used in combination with hemoglobin. Also very preferred the marker RLA-0 is used in combination with the hemoglobin-haptoglobin complex.

Diagnostic reagents in the field of specific binding assays, like immunoassays, usually are best provided in the form of a kit, which comprises the specific binding agent and the auxiliary reagents required to perform the assay. The present invention therefore also relates to an immunological kit comprising at least one specific binding agent for RLA-0 and auxiliary reagents for measurement of RLA-0.

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 thresh-hold over the entire range of data observed.

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.

In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity versus 1 - 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 - 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/-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.

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 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).

Clinical utility of the novel marker RLA-0 has been assessed in comparison to and in combination with the established marker hemoglobin using a receiver operator curve analysis (ROC; Zweig, M. H., and Campbell, G., Clin. Chem. 39 (1993) 561-577). This analysis has been based on well-defined patient cohorts consisting of 30 samples each from patients in T1-3; N0; M0, more progressed tumor, i.e., T4 and/or various severity of metastasis (N+ and/or M+), and healthy controls, respectively.

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 procedures set forth without departing from the spirit of the invention.

Description of the Figures

- Figure 1 Figure 1 shows a typical example of 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. Two distinctive spots are shown which both represent RLA-0. Both fit the theoretical size of 34.3 kDa; one spot fits the theoretical isoelectric point at pH 5.72, the other spot probably corresponds to phosphorylated RLA-0, most likely due to a post-translational modification. Each circle in the enlarged section of these gels indicates the position for the a RLA-0 protein form. RLA-0 protein was not detectable by the same method in healthy mucosa.
- Figure 2 Typical example of a Western-Blot. A polyacrylamide gel was loaded with tissue lysates from colorectal tumor tissue and adjacent healthy control tissue from 4 patients (subject 36: rectum ca (carcinoma), Dukes B; subject 37: rectum ca, Dukes A; subject 39: colon ca, Dukes A; and subject 40: colon ca, Dukes B) and after electrophoresis the proteins were blotted onto a nitrocellulose membrane. Presence of RLA-0 in the samples was tested using a polyclonal rabbit anti-RLA-0 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 "M". The lane containing recombinant RLA-0 is indicated by "300". The arrow indicates the position in the gel of the RLA-0 (RLA0) band. All tumor samples give a strong signal at the position of RLA-0, whereas only a weak signal can be detected in the lysates from adjacent normal control tissue.

Abbreviations

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

Example 1

Identification of RLA-0 as a potential colorectal cancer marker

25 Sources of tissue

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 In total, tissue specimen from 10 patients suffering from colorectal cancer are analyzed. From each patient three different tissue types are collected from therapeutic resections: 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

- 5 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 MgCl_2 , 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
10 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

- 15 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,
20 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
25 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)
30 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 RLA-0 as a potential marker for colorectal cancer

- 5 Each patient is analyzed separately by image analysis with the ProteomeWeaver® software (Definiens AG, Germany, München). In addition, all spots of the 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 each patient, 4 gels from the tumor sample are compared
10 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 RLA-0 is found to be specifically expressed or strongly overexpressed in tumor tissue and not detectable or less strongly expressed in healthy control tissue. It therefore – amongst many other proteins - qualifies as a candidate marker for use
15 in the diagnosis of colorectal cancer.

Example 2

Generation of antibodies to the colorectal cancer marker protein RLA-0

- Polyclonal antibody to the colorectal cancer marker protein RLA-0 is generated for further use of the antibody in the measurement of serum and plasma and blood
20 and stool levels of RLA-0 by immunodetection assays, e.g. Western Blotting and ELISA.

Recombinant protein expression in E. coli

- In order to generate antibodies to RLA-0, recombinant expression of the protein is performed for obtaining immunogens. The expression is done applying a
25 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.
30 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 RLA-0 protein is used. For Western-blot detection and later purification, the expressed protein contains a His-tag. The best expressing
5 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, 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
10 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-RLA-0 fusion protein is done following standard procedures on a Ni-chelate column. Briefly, 1 l of bacteria culture containing the expression vector
15 for the His-RLA-0 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
20 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 RLA-0

a) Immunization of mice

25 12 week old A/J mice are initially immunized intraperitoneally with 100 µg RLA-0. This is followed after 6 weeks by two further intraperitoneal immunizations at monthly intervals. In this process each mouse is administered 100 µg RLA-0 adsorbed to aluminum hydroxide and 10⁹ germs of *Bordetella pertussis*. Subsequently the last two immunizations are carried out intravenously on the 3rd
30 and 2nd day before fusion using 100 µg RLA-0 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. 1×10^8 spleen cells of the immunized mouse are mixed with 2×10^7 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 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. RLA-0-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

a) Immunization

For immunization, a fresh emulsion of the protein solution (100 µg/ml protein RLA-0) and complete Freund's adjuvant at the ratio of 1:1 is prepared. Each rabbit
5 is immunized with 1 ml of the emulsion at days 1, 7, 14 and 30, 60 and 90. Blood is drawn and resulting anti-RLA-0 serum used for further experiments as described in examples 3 and 4.

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

10 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
15 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).

20 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

25 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

5 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
10 IgG are collected. Monoclonal antibodies are labeled with digoxigenin according to the same procedure.

Example 3

Western Blotting for the detection of RLA-0 in human colorectal cancer tissue using polyclonal antibody as generated in Example 2.

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

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
20 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 PBS/0.05% Tween-20 and blocked with Roti®-Block blocking buffer (A151.1; Carl
25 Roth GmbH, Karlsruhe, Germany) for 2 h. The primary antibody, polyclonal rabbit anti-RLA-0 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 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-
30 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 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.

- 5 Results of a typical experiment are shown in Figure 2. The strong overexpression of RLA-0 in tumor tissue versus adjacent control tissue is found in 15 out of 16 subjects with colorectal cancer tested.

Example 4

ELISA for the measurement of RLA-0 in human stool specimen

10 a) Processing of stool specimen

- About 1 g of stool per sample is collected by the patients in a special sampling device from Sarstedt, Germany, Order-No. 80.623.022, frozen and stored at -20 °C. For analysis, the stool samples are thawed, tenfold diluted with a phosphate buffer, pH 7.4 and thoroughly mixed in order to yield a suspension of the stool sample in
15 the extraction buffer. After centrifugation for 15 min at 12,000 x g, the upper portion of the supernatant is saved for further analysis. An aliquot of this processed stool sample is used for quantification of RLA-0 by ELISA.

b) Measurement of RLA-0 from a processed stool sample

- For detection of RLA-0 in human a processed stool sample, a sandwich ELISA is
20 developed. For capture and detection of the antigen, aliquots of the anti-RLA-0 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-RLA-0 polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate,
25 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 stool samples from patients. After binding of RLA-0, plates are washed three times with 0.9% NaCl, 0.1% Tween-20. For specific

detection of bound RLA-0, wells are incubated with 100 µl of digoxigenylated anti-RLA-0 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
5 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,
10 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 stool samples obtained from well-
15 characterized patient cohorts, i.e., 30 patients having undergone colonoscopy and found to be free of adenoma or CRC, 30 patients diagnosed and staged as T1-3, N0, M0 of CRC, and 30 patients diagnosed with progressed CRC, having at least tumor infiltration in at least one proximal lymph node or more severe forms of metastasis, respectively. RLA-0 is measured as described above in a stool sample obtained from
20 each of these individuals. ROC-analysis is performed according to Zweig, M. H., and Campbell, *supra*. Discriminatory power for differentiating patients in the group T₁₋₃, N0, M0 from healthy individuals for the combination of RLA-0 with the established marker hemoglobin (Hämoglobin ELISA; Catalogue No.: K7816; Immundiagnostik AG, Wiesenstr. 4, D 64625 Bensheim, Germany) is calculated by
25 regularized discriminant analysis (Friedman, J. H., Regularized Discriminant Analysis, Journal of the American Statistical Association 84 (1989) 165-175).

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Patent Claims

1. A method for the diagnosis of colorectal cancer comprising the steps of
 - a) providing a stool sample obtained from an individual,
 - b) contacting said sample with a specific binding agent for 60S acidic
5 ribosomal protein P0 (RLA-0) under conditions appropriate for
formation of a complex between said binding agent and RLA-0, and
 - c) correlating the amount of complex formed in (b) to the diagnosis of
colorectal cancer.
2. Use of protein RLA-0 as a marker molecule in the diagnosis of colorectal
10 cancer from a stool sample obtained from an individual.
3. Use of protein RLA-0 as a marker molecule in the early diagnosis of colorectal
cancer from a stool sample obtained from an individual.
4. Use according to claim 3, wherein the early diagnosis is made with a sample
derived from CRC patients in the adenoma stage.
- 15 5. Use according to claim 3, wherein the early diagnosis is made with a sample
derived from CRC patients in stage T_{is} -3; N0; M0
6. Use of protein RLA-0 as a marker molecule for colorectal cancer in
combination with another marker molecule for colorectal cancer in the
diagnosis of colorectal cancer from a stool sample obtained from an
20 individual.
7. An immunological kit comprising at least one specific binding agent for
RLA-0 and auxiliary reagents for measurement of RLA-0.

Fig. 1

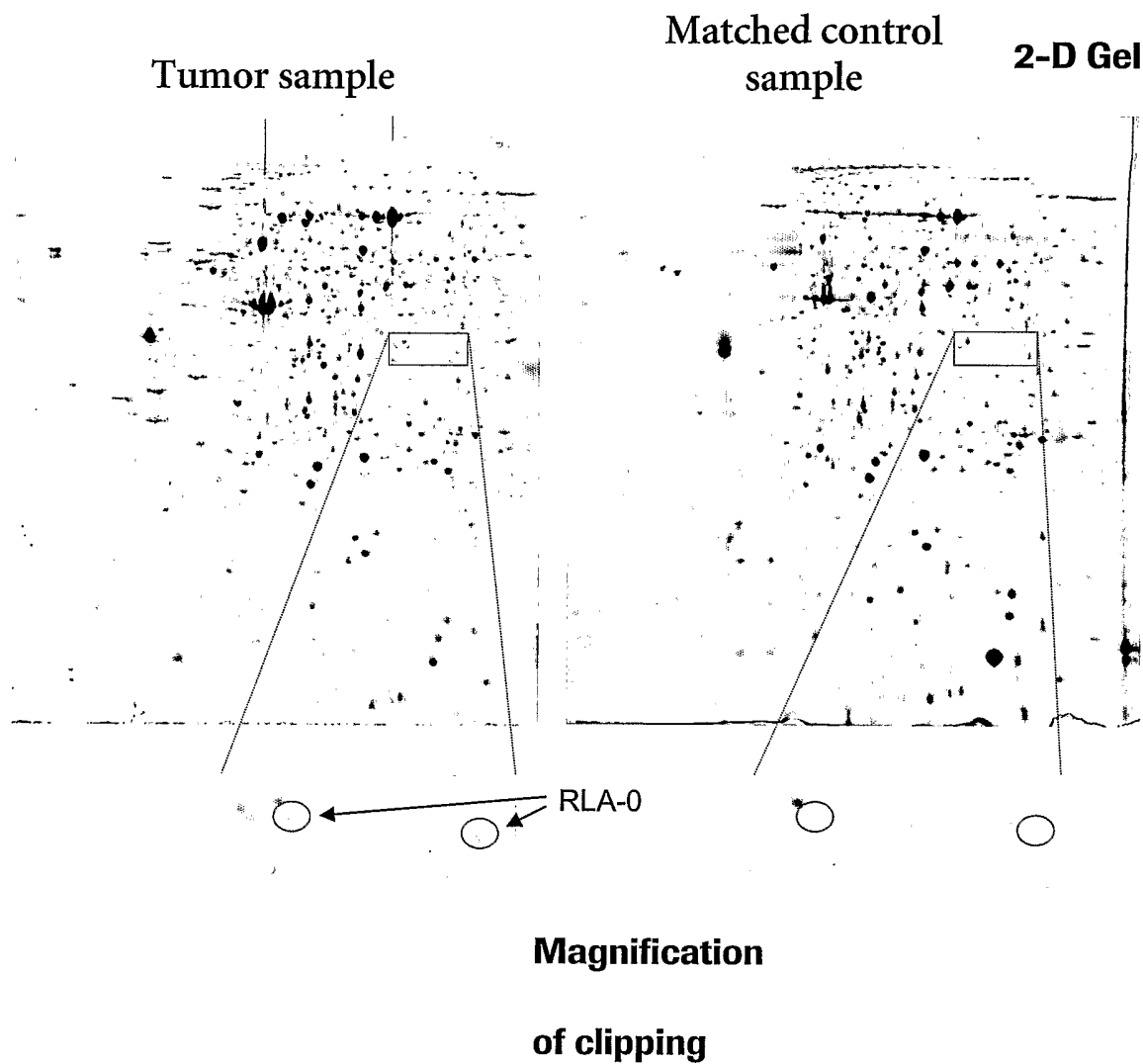
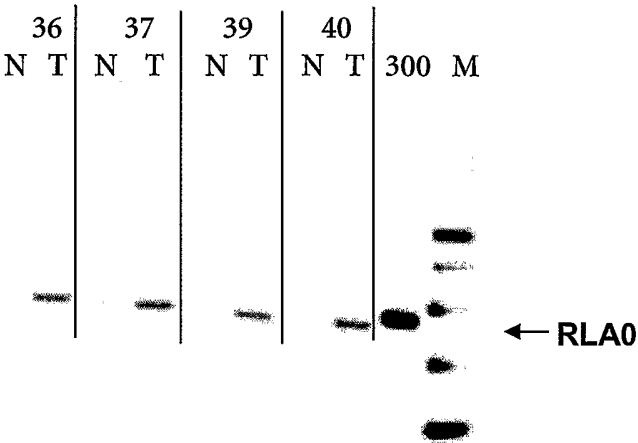


Fig. 2



- 1 -

SEQUENCE LISTING

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<120> Use of protein RLA-0 as a marker for colorectal cancer

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 65 70 75 80

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP2004/008763

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/574

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 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, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BARNARD G F ET AL: "INCREASED EXPRESSION OF HUMAN RIBOSOMAL PHOSPHOPROTEIN P0 MESSENGER RNA IN HEPATOCELLULAR CARCINOMA AND COLON CARCINOMA" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 52, no. 11, 1 June 1992 (1992-06-01), pages 3067-3072, XP001145714 ISSN: 0008-5472 the whole document ----- -/--	1-7

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Schalich, J

INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	POGUE-GEILE K ET AL: "RIBOSOMAL PROTEIN GENES ARE OVEREXPRESSED IN COLORECTAL CANCER ISOLATION OF A COMPLEMENTARY DNA CLONE ENCODING THE HUMAN S3 RIBOSOMAL PROTEIN" MOLECULAR AND CELLULAR BIOLOGY, vol. 11, no. 8, 1991, pages 3842-3849, XP002299999 ISSN: 0270-7306 the whole document -----	1-7