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(54) Titre : PROCEDE ET KIT POUR DIAGNOSTIC DE COLITE ULCEREUSE
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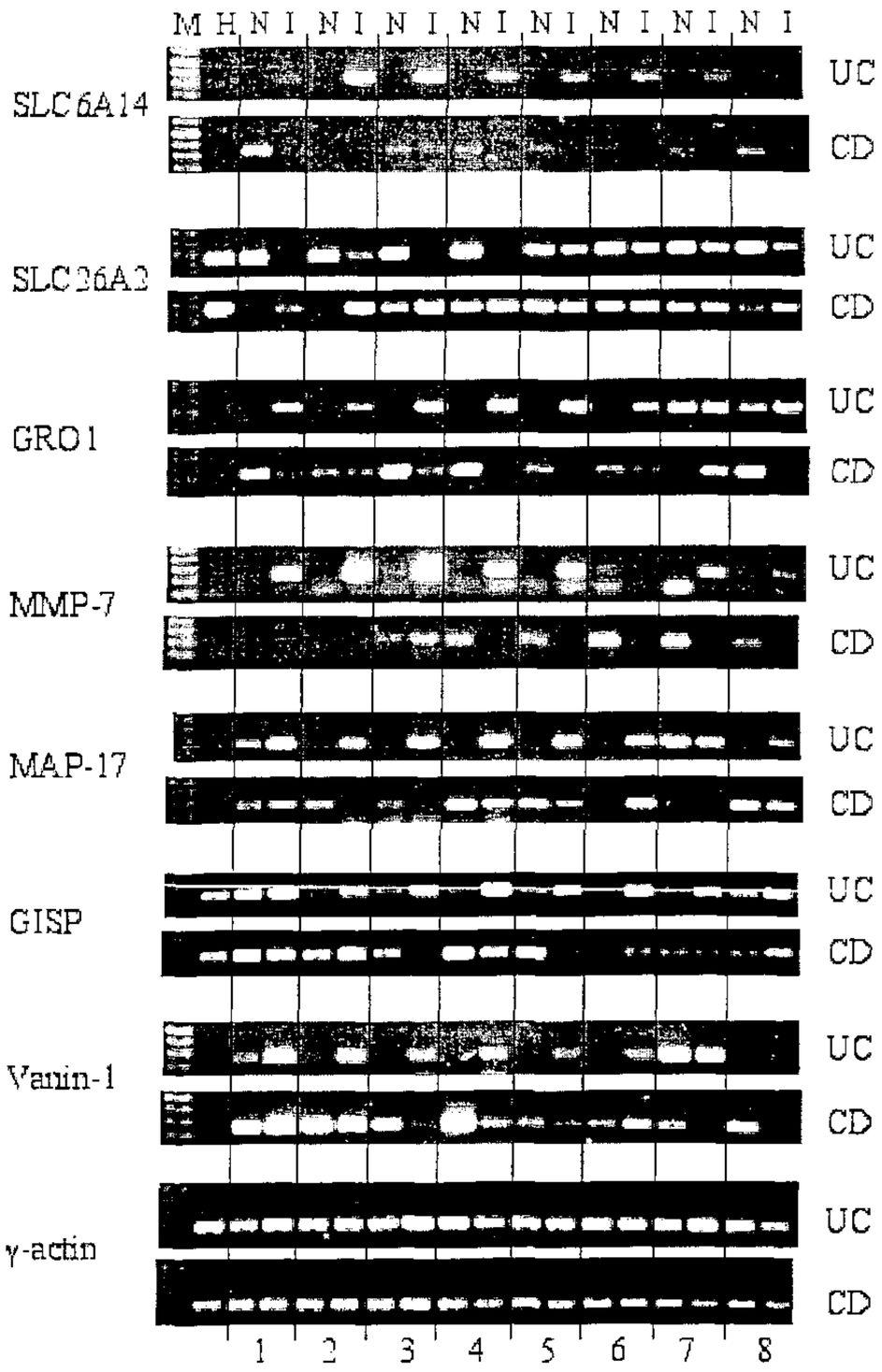

(57) Abrégé/Abstract:
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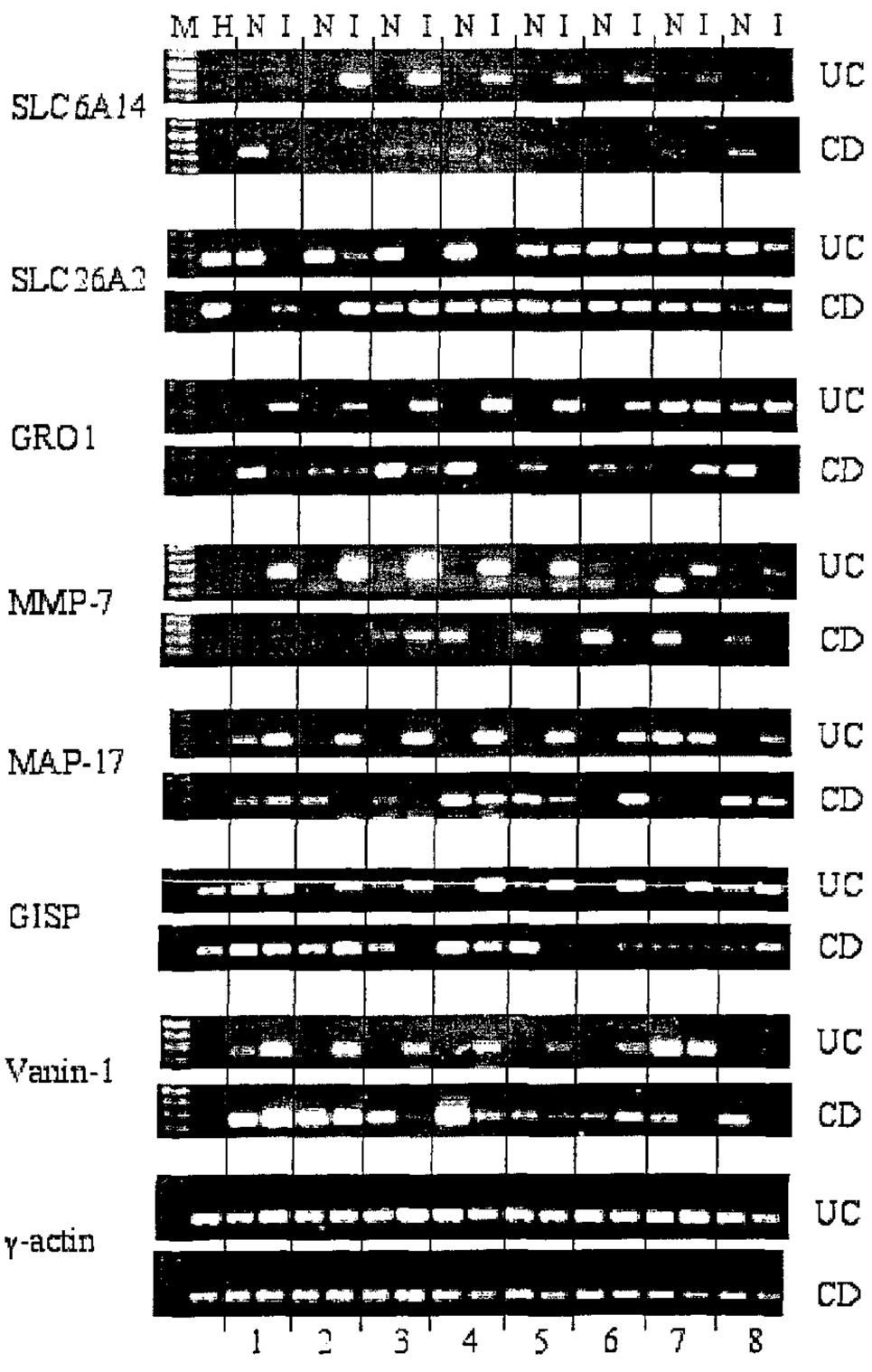

(57) Abstract: The differentiation between ulcerative colitis and Crohn's disease is made possible by a multi-gene approach where gene expression profiles in biopsy samples obtained from inflamed, and optionally also non-inflamed, areas in the intestines of a patient are studied.

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Method and kit for the diagnosis of ulcerative colitis

Field of the Invention

The present invention relates the diagnosis of inflammatory bowel diseases, and in particular to a method and kit for the prediction and/or diagnosis of ulcerative colitis. The invention discloses specific marker genes whose change in expression status, either collectively or a sub-set thereof, is indicative of ulcerative colitis. The present invention further relates to DNA-related methods by which quantification of the expression levels of said disease-associated marker genes directly from a biopsy allows for an immediate and accurate diagnostic test for disease type, and/or the assessment of the effect of a particular treatment regimen. The invention further discloses diagnostic kits for the detection of the expressions levels of said genes.

Background of the invention

Inflammatory bowel disease (IBD) is a term encompassing several conditions involving chronic inflammation in the gastrointestinal tract. Two of the most debilitating forms of IBD are ulcerative colitis (UC) and Crohn´s disease (CD). These diseases affect young people, with a typical debut at the age of 20-30 years, and disease management is a long-term commitment for both patient and physician, since there is currently no cure for either condition. Approximately 30% of IBD patients undergo surgery during their lifetime and patients with long-standing IBD are at considerable risk of developing colorectal cancer. Three out of ten IBD patients do not respond to the best available medical therapy today, even when high doses are used, causing considerable side effects.

Treatment of patients with active UC aims at reducing inflammation and promoting colon healing and mucosal recovery. The underlying cause of UC is not understood, nor is it known what triggers the disease to recur between its inactive and active forms. However, the active stage of the disease is characterised by significant inflammation of the mucosa, increased cell permeability, loss of protein and fluids. In severe stages deep inflammation of the bowel wall may develop with abdominal tenderness, tachycardia, fever and risk of bowel perforation.

One early symptom of ulcerative colitis is constipation with passage of blood or mucus in the stools. Several months or years may pass before diarrhoea develops with abdominal pain. Later symptoms include severe fatigue, weight loss, loss of appetite, fever and occasionally arthralgia.

The road to an established diagnosis of ulcerative colitis often includes a thorough study of the patient's medical history, the exclusion of other conditions, as well as several tests, e.g. blood tests, stool examination, barium enema X-ray, sigmoidoscopy, colonoscopy, and biopsy. The biopsy may be performed as part of a sigmoidoscopy or colonoscopy examination.

It is obvious that a possibility to clinically distinguish UC from colonic CD at an early stage would provide enormous benefits for both the patient and the physician. It would permit the design of accurate treatment regimes, prevent unnecessary medications and reduce treatment costs. Even though the overall clinical picture in IBD patients may show some clinically important differences between the major patient groups of UC and CD, there are substantial similarities, thus making it difficult for health care personnel to establish a correct diagnosis.

Prior art

The prior art indicates that the available methods for distinguishing between forms of IBD, and in particular the differentiation between UC and CD, apart from the above given examples of different examination procedures, have been focused on antibody based methods.

For example WO 03/036262 describes a method and apparatus for the differentiation of Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome, using the presence of faecal anti-*Saccharomyces cerevisiae* antibodies (ASCA) as a marker for Crohn's disease are provided. The apparatus includes an enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobins for the measurement of total endogenous ASCA in a human faecal sample. The method and apparatus may be used by healthcare providers to distinguish Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome.

WO 01/58927 describes diagnostic methods for detecting diseases associated with an autoantigen response to hTM in affected tissue, and in particular ulcerative colitis.

There remains a need for improved methods for the accurate, rapid and reliable diagnosis of ulcerative colitis, in particular in the context of distinguishing between ulcerative colitis and Crohn's disease in IBD patients.

One aim of the present invention is to make available such methods and kits for this purpose. One particular aim is to make available a method and kit which makes it possible to reach a reliable diagnosis at an early stage of the disease. Another aim is to make it possible to

distinguish between CD and UC also in difficult cases, where the clinical picture may be very similar.

Further aims underlying the invention, as well as the solutions offered by the invention and the associated advantages will become evident to a skilled person upon study of the description, examples and claims.

Summary of the invention

The present inventors have surprisingly found that the differentiation between ulcerative colitis and Crohn's disease is made possible by a multi-gene approach where the gene expression profiles in biopsy samples obtained from inflamed and optionally also non-inflamed areas in the intestines of a patient are studied.

The present invention is based on the discovery of potential marker genes which, either collectively or in sub-groups, are indicative of the human condition of ulcerative colitis (UC). The present inventors surprisingly found that quantification of the expression levels of a number of specific genes can be utilized in accurately and simply diagnosing from a biopsy, whether the patient is afflicted with the condition of UC or, for example, Crohn´s disease.

More specifically, methods are provided that allow nucleic acid amplification of seven (7) distinct genetic markers or sub-groups thereof, using pre-selected gene specific primers that allow for semi quantification of the expression levels of said genetic markers. The gene specific primers are designed to hybridise to opposing strands of the DNA encoding the genetic marker of interest such that though PCR amplification, a defined region of the encoding DNA of the genetic marker gene is produced. An assay and kit for the detection and monitoring expression status of said seven marker genes or sub-sets thereof in a biological sample are provided. The assay is a non-culture, PCR-based assay for the detection of said marker genes.

Brief description of the drawings

The present invention will be described in closer detail in the following description and examples, with reference to the drawings in which

Figure 1 shows the RT-PCR analysis of expression status of seven marker genes on biopsy samples from patients afflicted with either ulcerative colitis (UC) or with Crohn´s Disease (CD). The experimental protocol is out-lined in Example 6. (Key: M, is a base-pair marker, H, represents a biopsy from a totally normal healthy individual, I, represents a biopsy

sample taken from an inflamed area and N, represents a biopsy taken from a non-inflamed area from the same patient. Numbers at the bottom of the figure indicates patient number and the vertical black lines indicate an N and I biopsy sample derived from the same patient). Gamma actin was used as a loading control and indicates the expression status of a house-keeping gene used commonly to demonstrate equal mRNA input in all RT-PCR reactions.

Detailed description of the invention

Before the present invention is disclosed and described, it is to be understood that one skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations of the scope of the invention. Changes therein and other uses that will occur to those skilled in the art are encompassed within the spirit of the invention as defined by the scope of the claims.

As used herein, the term "complementary DNA primer" means an oligonucleotide, which anneals to the RNA template in a particular orientation to allow for the synthesis of a nascent DNA strand in the presence of reverse transcriptase in the biological sample under the conditions described herein.

Also as used herein, the "condition" under which a DNA strand is synthesized include the presence of nucleotides, cations and appropriate buffering agents in amounts and at temperatures, such that the RNA template and the DNA primer will anneal and oligonucleotides will be incorporated into a synthesized DNA strand if reverse transcriptase is not inhibited by the reverse transcriptase inhibitor drug. Exemplary conditions are set forth in the examples below. The described conditions have been optimised from other known RT/cDNA synthesis protocols. It is generally known that other conditions can be established for optimisation of a particular reverse transcriptase reaction on the basis of protocols well known to one of ordinary skill in the art.

As used herein, the term "primer pair" refers to two primers, one having a forward designation and the other having a reverse designation relative to their respective orientations on a double-stranded DNA molecule which consists of a sense and antisense sequence, such that under the amplification conditions described herein, the forward primer anneals to and primes amplification of the sense sequence and the reverse primer anneals to and primes

amplification of the antisense sequence. Primers can be selected for use in the amplification reaction on the basis of, having minimal complementarity with other primers in the reaction (to minimize the formation of primer dimers) and having Tm values with the range of reaction temperatures appropriate for the amplification method, preferably PCR. In addition, primers can be selected to anneal with specific regions of the RNA template such that the resulting DNA amplification product ranges in size from 100 to 500 base pairs in length and most preferably around 300 base pairs in length.

For example, in the conditions described above, the primer pair can consist of the oligonucleotide of SEQ ID NO: 13 as the forward primer and the oligonucleotide of SEQ ID NO: 14 as the reverse primer.

As used herein, the terms "detecting" or "detection" of the amplified DNA refers to qualitatively or quantitatively determining the presence of the amplified DNA strand, which is only synthesized if reverse transcriptase is resistant to the reverse transcriptase inhibitor drug added to the assay mixture. The amplification of the synthesized DNA can be detected by any method for the detection of DNA known in the art. For example, detection of the amplified DNA can be by Southern blot hybridisation assay, by visualization of DNA amplification products of specific molecular weight on ethidium bromide stained agarose gels, by measurement of the incorporation of radiolabeled nucleotides into the synthesized DNA strand by autoradiography or scintillation measurement.

The preferred detection method is by agarose gel electrophoresis using ethidium bromide staining and visualisation under UV light.

The principles of PCR and the conditions for amplification and detection of target nucleic acids are well known in the art and may be found in numerous references known to the skilled artisan, including, for example, U.S. 4,683,195; U.S. 4,683,202 and U.S. 4,965,188; all to Mullis *et al*. Briefly, a sample suspected of containing a target nucleic acid is heated to denature double-stranded nucleic acid in the presence of two oligonucleotide primers that are complementary to target sequences flanking the region to be amplified. The primers anneal to the separated target strands and are extended from each 3' hydroxyl end by a polymerising agent such as a thermostable polymerase. Double-stranded or single-stranded DNA can be amplified by PCR. RNA can also serve as a target by reverse transcribing RNA into cDNA.

The steps of denaturation, primer annealing and DNA synthesis are carried out at discrete temperatures, and repeated cycles result in exponential accumulation of the target nucleic acid. The PCR vessel is generally a stoppered plastic vessel or a cuvette or pouch as described in U.S. 5,229,297. Reagents for PCR amplification are typically mixed in a single vessel, and generally include primers, nucleoside triphosphates (generally dATP, dCTP, dGTP and dTTP or dUTP), thermostable DNA polymerase, magnesium containing buffer, and target nucleic acid. Reagents and conditions for PCR are well-known to one of ordinary skill in the art, and can be found, for example, in Guatelli *et al.* (1989) Clin. Microbiol. Rev. 2:217. For amplification of RNA targets, a reverse transcriptase may be utilized in addition to or in lieu of the thermostable DNA polymerase. Thermostable reverse transcriptase are particularly useful, as are thermostable DNA polymerases having reverse transcriptase activity. Methods for PCR amplification of RNA targets are known to one of ordinary skill in the art and described, for example, in U.S. 5,176,995, 5,310,652 and 5,322,770.

Detection of the DNA amplified by the PCR is generally conducted in a manner that the DNA is subjected to electrophoresis using agarose gel, acryl amide gel or the like, and then it is subjected to dyeing with nucleic acid-specific dyeing reagents. In the case of detecting double-stranded DNA, usually a fluorescent reagent such as ethidium bromide is allowed to enter between two strands of the DNA and then the fluorescent reagent is excited by an ultraviolet light source. As ethidium bromide entered between the two strands of the DNA emits fluorescence, detection is performed by means of capturing the fluorescence with a CCD camera or the like.

The object of the present invention is accomplished by the amplification, by PCR, of seven specific marker genes or sub-sets thereof and latter separation by electrophoresis of the products of this amplification, followed by appropriate colouring techniques that permit an adequate visualisation of the DNA in the gel including, but not limited to: colouring by silver salts, radioisotopes and enzymes combined with substrates that permit their detection

It is an object of certain embodiments of the present invention to provide a method of conducting nucleic acid amplification reactions in a single reaction chamber whereby internal primer pairs hybridise to opposing reigns of said target genetic marker genes, and amplification occurs by polymerase chain reaction.

Internal control primer pairs designed to hybridise to opposing strands of a suitable control housekeeping gene such that semi-quantitative comparisons can be made. Such a preferred housekeeping gene can be actin, GADPH, or elongation factors. By measuring the

intensity of the internal control signal and comparing that to the signals given by the said genetic marker genes, one can determine the degree of change of expression of said genetic marker gene from the normal levels of expression (*i.e.* those levels in which no disease state is present).

In the method of the present invention, PCR amplification is accomplished by pre-incubating all PCR reagents and a sample containing a target nucleic acid in the presence of appropriate gene specific primers and a thermostable polymerase enzyme. The resulting reaction mixture is cyclically heated under conditions allowing for the formation and amplification of primer extension products.

The reagents required for PCR are known to persons skilled in the art, and generally include at least two oligonucleotide primers that are sufficiently complementary to conserved regions of the target nucleic acid to hybridise thereto, four different nucleoside triphosphates, a thermostable polymerisation agent and any requisite cofactors for the polymerisation agent. Preferred nucleoside triphosphates are the deoxyribonucleoside triphosphates dATP, dCTP, dGTP and dTTP or dUTP, collectively termed dNTPs. Nucleoside triphosphates are commercially available.

Primers include naturally occurring or synthetically produced oligonucleotides capable of annealing to the target nucleic acid and acting as the point of initiation of nucleic acid synthesis under appropriate conditions, i.e., in the presence of nucleoside triphosphates, a polymerisation agent, suitable temperature, pH and buffer. The primers have sequences sufficiently complementary to the target nucleic acid to hybridise thereto, and are of sufficient length, typically from 10-60 nucleotides, to prime the synthesis of extension products in the presence of a polymerisation agent. Primers may be produced synthetically by automated synthesis by methods well known to one of ordinary skill in the art.

Design considerations for primers are well known in the art. Primers are selected to be substantially complementary to the sequences of the strands of the specific nucleic acid to be amplified, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for the extension product of the other primer. Preferably, the primers are exactly complementary with the target region. It is underlined that the primer pairs given in the present specification, examples and claims can be replaced by functionally equivalent primers, exhibiting specificity to the marker genes, without departing from the scope of the invention.

The inventors have unexpectedly identified seven (7) marker genes whose specific changes in expression status, collectively or in sub-sets, is indicative of the inflammatory bowel disease condition, UC. This opens the possibility of a rapid detection protocol at the molecular level designed to aid the examining physician in correctly predicating disease type.

The expression profile in inflamed and non-inflamed tissue is exemplified in Fig. 1 and in Table 1 below.

Table 1. Expression profiles in inflamed and non-inflamed tissue

Marker gene	Inflamed tissue		Non-inflamed tissue	
	Ulcerative colitis	Crohn's disease	Ulcerative colitis	Crohn's disease
SLC6A14	X	-	-	X
SLC26A2	-	X	XX	X
GRO1	X	-	-	X
MMP-7	XX	-	X	-
MAP-17	X	-	-	X
GISP	XX	X	X	XX
Vanin-1	X	(X)	-	X
Gamma actin (control)	X	X	X	X

X, and XX indicates the degree of expression; (X) indicates a result which is likely to be disregarded in light of the overall picture; - denotes lack of expression

The genetic markers are solute carrier family 6 member 14 (SLC6A14), solute carrier family 26 member 2 (SLC26A2), CXC chemokine growth-related oncogene-alpha (Gro-alpha) or (CXCL-1), Matrilysin also known as matrix metalloproteinase-7 (MMP-7), gastro-

intestinal secretory protein (GISP) also know as regenerating gene type IV (Reg IV), membrane associated protein 17 (MAP-17), and vanin-1. See Table 2.

Table 2. Target genetic marker genes

SEQ.ID.NO.1 (GeneBank Acc No NM-007231)

SEQ.ID.NO.2 (GeneBank Acc No NM-000112)

SEQ.ID.NO.3 (GeneBank Acc No NM-001511)

SEQ.ID.NO.4 (GeneBank Acc No BC003635)

SEQ.ID.NO.6 (GeneBank Acc No NM-005764)

SEQ.ID.NO.6 (GeneBank Acc No BC017089)

SEQ.ID.NO.7 (GeneBank Acc No NM-004666)

Polymerisation agents are compounds that function to accomplish the synthesis of the primer extension products. The polymerisation agents are thermostable, i.e., not permanently inactivated when heated for brief periods to temperatures typically used in PCR for denaturation of DNA strands, e.g., 93-95 °C, and are preferentially active at high temperatures. In a preferred embodiment the polymerisation agent is a thermostable DNA polymerase, including, for example, DNA polymerase obtained from thermophilic bacteria such as, *Thermococcus litoralis, Bacillus stearothermophilus, Methanothermus fervidus, Thermus aquaticus, T. filiformis, T. flavus, T. lacteus, T. rubens, T. ruber* and *T. thermophilus*; or from thermophilic archaebacteria such as *Desulfurococcus mobilis*, *Methanobacterium thermoautotrophilcum, Sulfolobus solfataricus, S. acidocaldarius* and *Thermoplasma acidophilum*. In a most preferred embodiment, the polymerisation agent is *Thermus aquaticus* (Taq) polymerase, *T. thermophilus* (Tth) polymerase or *Thermococcus litoralis* polymerase. Thermostable reverse transcriptase and DNA polymerases having reverse transcriptase activity are also contemplated as polymerisation agents.

The thermostable polymerases may be obtained commercially or by methods known in the art. In particular, Taq polymerase is available commercially in recombinant and native form (Perkin Elmer-Cetus) or can be produced by the method described by Lawyer et al., (1989) or in U.S. 4,889,818. Tth polymerase is commercially available from Finnzyme Co.,

Finland and from Toyobo Co., Japan. *Thermococcus litoralis* polymerase is commercially available from New England Biolabs and can be produced by the method described in U.S. 5,322,785.

Antibodies specific for the thermostable polymerisation agents may be included in the pre-amplification step to inhibit the polymerisation agent prior to amplification. Antibodies can be produced by methods known to one of ordinary skill in the art and found, for example, in Harlowe et al. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor, NY. In accordance with the present invention, the term antibodies includes monoclonal and polyclonal antibodies produced by conventional methodologies, recombinantly produced antibodies, and chemically or recombinantly produced fragments of antibodies, such as Fab fragments. In a preferred embodiment, the antibodies are monoclonal.

In a preferred embodiment of the present invention, the antibody is a monoclonal antibody against Taq polymerase, Tth polymerase, or *Thermococcus litoralis* polymerase. In a more preferred embodiment, the antibody is a monoclonal antibody against Taq polymerase. Monoclonal antibodies against Taq polymerase are known in the art and described, for example, in U.S. 5,338,671. In accordance with the present invention, antibodies defined as specific for polymerisation agent are those antibodies that are capable of inhibiting the enzymatic activity of the polymerisation agent at temperatures from about 20-40 °C. The antibodies of the invention are inactivated by elevated temperatures used during PCR thermal cycling. The ability of the antibodies to inhibit enzymatic activity of the polymerase can be determined by assays known to one of ordinary skill in the art, as described, for example, by Sharkey et al., (1994).

The present invention provides a method for the amplification of a target nucleic acid, and optionally, the subsequent detection of the nucleic acid, in a sample suspected of containing the target nucleic acid. The sample may be any sample suspected of containing a target nucleic acid, including, for example, a tissue sample, blood, hair, body fluid, bacteria, virus, fungus, bacterial infected cell, virally infected cell, and so on. The target nucleic acid may be DNA or RNA. A sufficient number of bases at both ends of the sequence to be amplified must be known in order to design primers capable of hybridising to the different strands of the target nucleic acid at suitable positions for PCR amplification. The target nucleic acid may be extracted or partially extracted from the tissue sample prior to PCR, for example, by removing proteins or cellular material from the sample. Methods for extracting

nucleic acids from samples are known to one of ordinary skill in the art and may be found, for example, in Sambrook *et al.*, (1989) and Saiki *et al.*, (1985).

In a preferred embodiment, biopsies resected from the gastro-intestinal tract and from area believed to be exhibiting signs of the disease is particularly preferred as the source of material.

In the method of amplification of the present invention, the sample or a preparation of nucleic acids extracted from the sample is contacted with the reagents typically used for PCR, including at least two oligonucleotide primers modified to contain at least one phosphorothioate linkage, four different nucleoside triphosphates, a thermostable polymerisation agent, and an appropriate buffer, and further with an exonuclease to form a reaction admixture. In another embodiment, an antibody specific for the polymerisation agent is included in the admixture.

The conventional PCR reagents, including primers, nucleoside triphosphates, polymerisation agent, and appropriate buffer are utilized at concentrations generally appropriate for PCR and known to one of ordinary- skill in the art. In a preferred embodiment, the nucleoside triphosphates are dATP, dCTP, dGTP and dTTP. In a preferred embodiment the polymerisation agent is a thermostable DNA polymerase. Preferred DNA polymerases are Taq polymerase, Tth polymerase and *Thermococcus litoralis* polymerase. Taq polymerase is particularly preferred.

The amplification method is preferably conducted in a continuous, automated manner. Appropriate instrumentation for automated PCR is well-known to the ordinarily skilled artisan and described, for example, in U.S. Patent Nos. 4,965,188, 5,089,233 and 5,229,297. The skilled artisan can also easily detect amplified product, for example, by separating PCR products by agarose gel electrophoresis and visualizing by ethidium bromide staining, or detecting by hybridisation with a labeled probe capable of hybridising with the amplified nucleic acid or a variety of other detection methods well-known to one of ordinary skill in the art.

One embodiment of the invention is thus a method for the differentiation between ulcerative colitis and Crohn's disease based on the analysis of gene expression profiles in biopsy samples obtained from inflamed and non-inflamed areas in the intestines of a patient, wherein the expression levels of at least two of a number of marker genes are determined, said

at least two marker genes chosen among SEQ.ID.NO. 1, SEQ.ID.NO. 2, SEQ.ID.NO. 3, SEQ.ID.NO. 4, SEQ.ID.NO. 5, SEQ.ID.NO. 6, and SEQ.ID.NO. 7.

Another embodiment of the invention is a method wherein the expression levels of SEQ.ID.NO. 1 and SEQ.ID.NO. 2 are determined and wherein the expression of SEQ.ID.NO. 1 in inflamed tissue and lack of expression in non-inflamed tissue, together with the lack of expression of SEQ.ID.NO. 2 in inflamed tissue is taken as an indication of ulcerative colitis.

A third embodiment of the invention is a method wherein the expression levels of SEQ.ID.NO. 1, SEQ.ID.NO. 2 and SEQ.ID.NO. 3 are determined, and wherein the expression of SEQ.ID.NO. 1 in inflamed tissue and lack of expression in non-inflamed tissue, together with the lack of expression of SEQ.ID.NO. 2 in inflamed tissue, and the expression of SEQ. ID.NO. 3 in inflamed tissue and lack of expression in non-inflamed tissue, is taken as an indication of ulcerative colitis.

A fourth embodiment is a method wherein the expression levels of SEQ.ID.NO. 1, SEQ.ID.NO. 2 and SEQ.ID.NO. 4 are determined, and wherein the expression of SEQ.ID.NO. 1 in inflamed tissue and lack of expression in non-inflamed tissue, together with the lack of expression of SEQ.ID.NO. 2 in inflamed tissue, and the expression of SEQ. ID.NO. 4 in inflamed tissue and lack of expression in non-inflamed tissue, is taken as an indication of ulcerative colitis.

A fifth embodiment is a method wherein the expression levels of SEQ.ID.NO. 1 through 7 are determined, and wherein the expression of SEQ.ID.NO. 1, 3, 4, 5, 6, and 7 in inflamed tissue and lack of expression in non-inflamed tissue, together with the lack of expression of SEQ.ID.NO. 2 in inflamed tissue, is taken as an indication of ulcerative colitis.

According to a preferred embodiment, the method of any one of the above embodiments includes a step wherein the expression level of each marker gene is determined through nucleic acid amplification of said genes using gene specific primers, and determination of the amplification results. The nucleic acid amplification is preferably performed using PCR and the gene specific primers preferably chosen among SEQ.ID.NO. 13 – 26.

The determination of the amplification results is preferably performed using ethidium bromide staining and visualisation under UV light.

The present invention further provides a kit for PCR comprising, in the same or separate containers, a thermostable polymerisation agent, and primer pairs designed to allow PCR amplification of said target genes. Additional containers can also be provided for the

inclusion of, for example, additional antibodies specific to the PCR polymerisation agent and reagents for PCR, including, for example, nucleoside triphosphates, primers and buffers.

Consequently, the present invention makes available a kit for the differentiation between ulcerative colitis and Crohn's disease based on the analysis of gene expression profiles in biopsy samples obtained from inflamed and non-inflamed areas in the intestines of a patient, said kit including gene specific primer pairs directed to at least two marker genes chosen among SEQ.ID.NO. 1, SEQ.ID.NO. 2, SEQ.ID.NO. 3, SEQ.ID.NO. 4, SEQ.ID.NO. 5, SEQ.ID.NO. 6, and SEQ.ID.NO. 7.

Said gene specific primer pairs are preferably chosen among SEQ.ID.NO. 13 and SEQ.ID.NO. 14; SEQ.ID.NO. 15 and SEQ.ID.NO. 16; SEQ.ID.NO. 17 and SEQ.ID.NO. 18; SEQ.ID.NO. 19 and SEQ.ID.NO. 20; SEQ.ID.NO. 21 and SEQ.ID.NO. 22; SEQ.ID.NO. 23 and SEQ.ID.NO. 24; and SEQ.ID.NO. 25 and SEQ.ID.NO. 26.

According to one embodiment of the invention, said specific primers are SEQ.ID.NO. 13 and SEQ.ID.NO. 14; SEQ.ID.NO. 15 and SEQ.ID.NO. 16; and SEQ.ID.NO. 17 and SEQ.ID.NO. 18.

According to another embodiment of the invention, said specific primers are SEQ.ID.NO. 13 and SEQ.ID.NO. 14; SEQ.ID.NO. 15 and SEQ.ID.NO. 16; and SEQ.ID.NO. 19 and SEQ.ID.NO. 20.

The kit according to the invention further preferably comprises a thermostable polymerisation agent and requisite cofactor(-s). In a preferred embodiment the polymerisation agent is a DNA polymerase. In a more preferred embodiment the polymerase is Taq polymerase, Tth polymerase, or *Thermococcus litoralis* polymerase. Taq polymerase is particularly preferred. The preferred antibody is a monoclonal antibody specific for Taq polymerase.

The oligonucleotide primers preferably are 19-25 nucleotides in length and are designed as primer-pairs that will under standard conditions anneal to the target DNA of said genetic marker genes. In this case seven primer pairs are provided that will allow successful amplification of the seven said genetic marker genes. The specific primers used are presented in Table 3.

Table 3. Primer-pairs

SLC6A14 (for) 5′- GTG CTG AGA TTA CAG GTG TGA GCC-3′ (SEQ.ID.NO.13)

SLC6A14 (rev) 5′ -CCC TTC ACA CCT CCC CCA ATT AGA-3′ (SEQ.ID.NO.14)

SLC26A2 (for) 5′- GTG GAG AGA GGG AAA GAA TGT TGC-3′ (SEQ.ID.NO.15)

SLC26A2 (rev) 5′- CCA GTT TAG GAC AGA TTC CAT GGG-3′ (SEQ.ID.NO.16)

GRO1 (for) 5′- GTG CCT AAT GTG TTT GAG CAT CGC-3′ (SEQ.ID.NO.17)

GRO1 (rev) 5′- GCC CCT TTG TTC TAA GCC AGA AAC-3′ (SEQ.ID.NO.18)

MMP7 (for) 5′- CAG GCA GAA CAT CCA TTC ATT CAT TC-3′ (SEQ.ID.N0.19)

MMP7 (rev) 5′- GAC ATC TAC CCA CTG CAA GTA TAG-3′ (SEQ.ID.NO.20)

MAP17 (for) 5′- CCG TCG GAA ACA AGG CAG ATG GAG-3′ (SEQ.ID.NO.21)

MAP17 (rev) 5′- GAA GGA CGT GTG AGC AGG ATG GGA-3′ (SEQ.ID.NO.22)

GISP (for) 5′- GGT GGG AAC AAG CAC TGT GCT GAG-3′ (SEQ.ID.NO.23)

GISP (rev) 5′- GGC TGG AGA TGC ACT CTT CTA GAC-3′ (SEQ.ID.NO.24)

Vanin1 (for) 5′- GCC AGC AAA ACA TCA TTT TGA GAC-3′ (SEQ.ID.NO.25)

Vanin1 (rev) 5′- GCC TAT CAC CAA CAC ATC AAT ATG-3′ (SEQ.ID.NO.26)

The genetic markers disclosed are, solute carrier family 6 member 14 (SLC6A14), as given by SEQ.ID.NO.1, solute carrier family 26 member 2 (SLC26A2) as given by SEQ.ID.NO.2, CXC chemokine growth-related oncogene-alpha (Gro-alpha) or (CXCL-1) as given by SEQ.ID.NO.3, Matrilysin also known as matrix metalloproteinase-7 (MMP-7) as given by SEQ.ID.NO.4, gastro-intestinal secretory protein (GISP) also know as regenerating gene type IV (Reg IV) as given by SEQ.ID.NO.5, membrane associated protein 17 (MAP-17) as given by SEQ.ID.NO.6, and Vanin-1 as given by SEQ.ID.NO.7. See Table 2, *supra*.

In the methods illustrated by the examples, a number of method based sequences were used. These are presented in Table 4.

Table 4. Method based sequences

(SEQ. ID. NO. 8.) 5'-TAG TCT ATG ATC GTC GAC GGC TGA TGA AGC GGC CGC TGG AGT TTT TTT TTT TTT TTT TTV-3'

(SEQ. ID. NO. 9) 5'-TGA TGA AGC GGC CGC TGG-3'

(SEQ. ID. NO. 10) 5'- TTC ATC AGC CGT CGA CGA TC –3'

(SEQ. ID. NO. 11) 5'-CGT AAG CTT GGA TCC TCT AGA GC-3'

(SEQ. ID. NO. 12) 5'-TGC AGG TAC CGG TCC GGA ATT CC-3'

Solute carrier (SLC) proteins comprise of a very large family of energy dependent transport molecules and have critical physiological roles in nutrient transport and may be utilized as a mechanism to increase drug absorption. However, there is limited understanding of these proteins at the molecular level due to the absence of high-resolution crystal structures.

In total, 1-2% of adults and 6-8% of children suffering from kidney stones have cystinuria, a defect in the transport of amino acids, which leads to high concentrations of cystine in the urine. Two genes have been implicated, solute carrier family 3 (cystine, basic and) neutral amino acid transporter, member 1 (SLC3A1) coding for the protein related to the system of amino-acid transporter, and solute carrier family 7, member 9 (SLC7A9). Both of these solute carriers are believed to be involved in stone formation which may ultimately lead to urinary tract infection and, eventually, renal failure.

The inventors have identified two known solute carriers (SLC6A14 and SLC26A2) whose expression is significantly altered in IBD. To the best knowledge of the inventors, this is the first reporting of the potential involvement of solute carriers in inflammatory bowel diseases. It is therefore a novel finding that solute carriers might contribute to the pathogenesis of IBD.

CXC chemokine growth-related oncogene-alpha (Gro-alpha also known as GRO1) is as described a cytokine and as such can alter the migratory responses of numerous cell types in local areas of inflammation. It and has been described to be over expressed in human inflamed corneas (Spandau *et al.*, 2003) and in addition, it has also been shown that rats chemically induced to exhibit inflammation of the gut show up-regulated levels of GRO1 (Hirata *et al.*, 2001). Using a cDNA microarray approach, Heller *et al.*, 1997 describes novel

participation of the, chemokine Gro alpha in rheumatoid arthritis and inflammatory bowel disease, however the invention presented here describes, in the inventors best knowledge for the first time, that GRO1 while over expressed in conditions of UC is down regulated in conditions of CD. While it is described in Isaacs et al, 1992, that expression of GRO1 in UC is higher than that seen in CD, here it has been demonstrated that there exists an inverse correlation of UC verses CD with respect to GRO1 expression levels. Lastly Lawrence *et al.*, 2001 describes identifying GRO 1 as being up-regulated in UC, but the design of the study was such that biopsy samples where pooled before analysis, therefore is was not possible to know whether GRO1 was up-regulated in more than 1 patient.

Matrilysin or (matrix metalloproteinase-7) was first discovered in the involuting rat uterus; it has also been known as uterine metalloproteinase, putative metalloproteinase (Pump-1), and matrix metalloproteinase 7 (MMP-7). It is the smallest member (28 kDa) of a family of 15 MMPs that together are able to degrade most of the macromolecules of the extra cellular matrix. This family is briefly reviewed; all members are zinc metalloproteinases that occur in zymogene form with the active site zinc blocked by cysteine. Matrilysin can degrade a wide range of gelatins, proteoglycans, and glycoproteins of the matrix and can activate several other MMPs including collagenase (reviewed in Woessner, 1996).

It is frequently expressed in various types of cancer including colon, stomach, prostate, and brain cancers. Previous studies have suggested that matrilysin plays important roles in the progression and metastasis of colon cancer. Recently it has been described by Newell *et al.*, 2002 that there is an increase of matrilysin expression at different stages of UC-associated neoplasia. This work however does not determine whether such increased expression is a result of UC or rather due to the presence of neoplasia.

Membrane associated protein 17 (MAP-17) or otherwise know as DD96, is a small protein has to date no described function. Regarding GISP, again there is little known. In both cases neither has been described as potentially involved in inflammation.

Pantetheinase (EC 3.5.1.) is an ubiquitous enzyme which in vitro has been shown to recycle pantothenic acid (vitamin B5) and to produce cysteamine, a potent anti-oxidant. The enzyme is encoded by the Vanin-1 gene and is widely expressed in mouse tissues. Vanin-1 is a GPI-anchored pantetheinase, and consequently an ectoenzyme. It has been suggested that Vanin/pantetheinase might be involved in the regulation of some immune functions maybe in the context of the response to oxidative stress (Pitari *et al.*, 2000).

To the best knowledge of the inventors, this is the first description of the potential role of Vanin-1 in IBD.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples are only intended to illustrate the invention and not to limit the same. While they are typical of methods and method steps that might be used, others known to those skilled in the art may be adopted without resorting to undue experimentation.

Examples

Example 1. Collection of biopsy material

The biopsies were taken from patients who were selected on the basis of clinical and pathological evidence of having the inflammatory condition of CD or UC. A total of three biopsies were collected from an inflamed site in the colon, together with three biopsy samples from a non-inflamed region of a single individual patient. This was done for a total of 16 different patients of which eight were diagnosed for CD (patient 1-8) and eight for UC (patient 9-16). The UC patient group comprised 2 females and 6 males, the age range being 29-77 years. The CD age group correspondingly 3 females and 5 males, age range 27 - 59

The biopsies from each anatomical site of one patient were pooled and total RNA isolated using Quiagen Rneasy Kit and a Pellet Pestel Motor Homogenizer according to the manufacturer's protocol. In this way 32 samples of total RNA were isolated, two samples per patient: inflamed (target) and non-inflamed (control).

Example 2. Performing cDNA synthesis of the RNA

Two microgram of each RNA sample (32 in total) was used for a first strand cDNA synthesis using 10pM of the Oligo-dT-primer dT-joint (5'-TAG TCT ATG ATC GTC GAC GGC TGA TGA AGC GGC CGC TGG AGT TTT TTT TTT TTT TTT TTV-3' (SEQ. ID. NO. 8.) introducing to every synthesised cDNA molecule three restriction enzyme cutting sites: SalI, NotI and BpmI. The buffer, desoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and the enzyme reverse transcriptase (Superscript II) were taken from Gibco BRL and the reactions were performed according to the manufactures guidelines. The reaction mixture for first strand synthesis excluding the enzyme was pre-incubated for 5 min at 65°C in a PCR machine (PCR sprint from Hybaid), chilled on ice, and then preheated to 42°C,

before the enzyme Superscript II was added and incubated for 1h at 42°C in a PCR machine (PCR sprint from Hybaid).

For the second strand synthesis, 41ul second strand buffer mix were added to the reactions according to the provided protocol (Gibco BRL) and 4µl E.coli Polymerase I (New England Biolabs), 1.5 µl E.coli DNA ligase (New England Biolabs) and 0.7 µl Rnase H (Gibco BRL) in a total volume of 160 µl. The reactions were incubated for 2.5h at 16°C in the PCR machine PCRsprint and then purified using the Quiagen PCR Purification Kit according to the protocol provided. Every sample (32 in total) was eluted with 32 µl of elution buffer and 26 µl of each sample was used for the following steps.

Example 3. Amplification of the 3'-termini of the cDNAs

Due to limited amounts of material obtained from such biopsies, a pre-amplification step was necessary. For in vitro amplification of the 3'-end of cDNAs, 26 µl of cDNA from every sample was digested with 10U of the restriction enzyme DpnII in a volume of 30 µl for 3h at 37°C. The cut cDNAs were purified once more using Quiagen PCR purification Kit and the cDNAs were eluted in 47 µl elution buffer. The following circular ligation step was performed in a volume of 50 µl including 44 µl of the DpnII cut cDNA and 2000U T4 DNA ligase (New England Biolabs). These reaction mixtures were incubated at 22°C for 1h, heat inactivated by 65°C for 10 min and 25 µl of each reaction mixture was used for the amplification step. A mixture for 5 reactions per sample was put together (5x 50 µl= 250 µl in total) containing 25 µl cDNA (DpnII cut and circular ligated), 25 µl 10x Advantage 2 PCR buffer (Clontech), 5 µl joint-Not primer (10 pmol/µl; 5'-TGA TGA AGC GGC CGC TGG-3'(SEQ. ID. NO. 9)), 5 µl joint-Sal primer (10pmol/µl; 5'- TTC ATC AGC CGT CGA CGA TC –3' (SEQ. ID. NO. 10), 5 µl 10mM dNTP mix and 5 µl 50x Advantage 2 Taq-Polymerase (Clontech). For each sample the mix was distributed into 5 PCR reaction tubes and PCR performed under the following conditions: 1 min 94°C then 16x (20 sec 94°C, 20 sec 55°C, 1 min 72°C).

Four of the reactions per sample were removed and placed on ice and the optimal cycle number was determined with one of the reactions per sample. The optimal cycle number was determined to 18 cycles for all 32 samples, thus for the remaining four reactions per sample two additional cycles [2x (20 sec 94°C, 20 sec 55°C, 1 min 72°C)] were performed. The 4 PCR reactions per sample were subsequently purified using the Quiagen PCR purification Kit.

For the purification, the four reactions per sample were pooled (total of 200 µl) and then eluted with 34 µl elution buffer. The purified reactions were the starting material for the identification of the differentially expressed genes protocol.

Example 4. Isolation of the differentially expressed cDNA (subtraction protocol) from human biopsies

Isolation of differentially expressed cDNAs was performed according to the protocol outlined in (von Stein O. D., 2001) with minor modifications to the protocol.

Example 5. Screening for the differentially expressed genes

Upon construction of a cDNA library, 2.000 clones were plated out from each subtraction on one 22 cm^2 agar plate. From these plates 384 colonies were picked and placed in 384 well plates with 70 µl LB medium/well (see Maniatis *et al.*, Molecular cloning laboratory book, Appendix A.1) (+ ampicillin 100 mg/ml) using BioPick machine of BioRobotics (Cambridge, UK). The bacterial clones were incubated over night at 37°C and then used for colony PCR. This PCR was performed in 384 PCR well plates in a volume of 20 µl per sample. One PCR reaction included: 2 µl 10x PCR buffer, 0,4 µl Sport-Not primer (10 pmol 5'-CGT AAG CTT GGA TCC TCT AGA GC-3' (SEQ. ID. NO. 11), 0,4 µl of Sport-Sal primer (10 pmol 5'-TGC AGG TAC CGG TCC GGA ATT CC-3' (SEQ. ID. NO. 12)), 1,6 µl dNTP mix (25 mM each), 0,4 µl 0,1% Bromphenol blue and 0,5 µl DynAzyme Taq-polymerase (2 U/µl; Finnzyme). A master mix for all reactions was prepared, distributed and then inoculated with a 384 plastic replica. The PCR cycling parameters were: 2 min 94°C, 37 times (30 sec 94°C; 30 sec 50°C, 1 min 72°C) and 5 min 72°C.

Following amplification, PCR reactions were spotted on Hybond N+ membrane (Amersham) using Microgrid TAS of BioRobotics. All clones were spotted in duplicate and genomic DNA was used as guide dots. On one filter 384 genes of all four subtractions were positioned. 24 duplicates were made for analyses by hybridisation with different radioactive cDNA probes.

These filters were then hybridised with the radioactive labeled subtracted cDNAs of all eight patients. Sixteen filters were used in 16 different hybridisation experiments. For that 1µl

of the cDNAs were used for the labelling with Klenow polymerase. The hybridisation protocol was that of Church-protocol as outlined in (Maxam and Gilbert 1984).

Phospho-imager Fuji film BAS 1800II with BAS 1800 III R program and Array vision version 6.0 (Imaging Research Inc) were used to determine the degree of differentially expression. Genes which were differentially expressed in at least three of the eight patients with an induction or reduction rate of three fold were sequenced and BLAST analysis performed to identify these isolated differentially expressed genes.

Example 6. Confirmation of true differential expression

Several genes showed strong dysregulation during these analyses. To confirm these data RT-PCR were performed using gene-specific oligonucleotides and un-amplified cDNA material derived from the same eight patients. Approximately, 2µg total RNA of inflamed and non-inflamed tissue (same as used for the subtraction) were taken for a first strand cDNA synthesis as described in example 5. After the cDNA synthesis the samples were incubated for 3min at 96°C and then 1:10 diluted with distilled water.

10 µl of further 1:10 dilutions were taken for one 50 µl PCR reaction. The PCR reaction included: 5 µl 10x PCR buffer, 1 µl forward primer (10 pmol/µl) and1 µl reverse primer (10 pmol/µl) of the specific genes (SEQ. ID. NO. 1 - 7)), 0.5 µl dNTP mix (25 mM each) and 0,5 µl DynAzyme Taq-polymerase (2 U/µl; Finnzyme). A master mix minus the cDNA for the reactions was prepared, distributed and then the cDNA added. The PCR cycling parameters were: 1 min 94°C, 26-35 times (30 sec 94°C; 30 sec 55°C, 1 min 72°C) and 5 min 72°C. The cycle number was dependent which gene fragments were amplified. The primer pairs were those shown in Table 3.

These analyses lead to the identification of seven (7) genetic markers whose change in expression status, collectively or in sub-sets, when compared to normal tissue would allow for a correct predication rate of over 90% with regard to UC. To confirm these findings, the markers where further screened against a larger sample collection of biopsies.

Example 7. High throughput screening

To confirm the preliminary results of the RT-PCR using the cDNA of eight UC patients and three CD patients (see Example 6), it was decided to analyse the expression of all isolated

genes in a large scale. For that purpose, all genes that could be isolated from the screening were spotted on a Hybond N+ membrane (Amersham) using the Microgrid TAS (BioRobotics). As described in Example 5 the genes were amplified via a colony PCR for the spotting.

This master filter membrane was then synthesized 240 times and hybridised with radioactive labelled cDNA deriving from biopsies from 50 individual UC patients and 50 individual CD patients. The biopsies were taken from the inflamed and non-inflamed area of the patients in the left side of the colon. As a baseline control the biopsies deriving from the left side of the colon of five healthy people were pooled.

Example 8. Verification through Blind Study

To provide greater statistical weight, it was necessary to perform expression analyse of the seven genetic markers on "blind" biopsy samples, whereby it was not known whether the biopsy was derived from a patient suffering from UC or CD. As described previously, RT-PCR analysis of the said genetic markers was performed and by combining the total picture of expression patterns resulting from said genetic markers it was possible to determine with over 90% certainty the correct form of IBD.

Analysis of the results (Blind study) show that already the combination of SEQ. ID. NO. 1 and 2 give a reliable result, whereas the combination of SEQ. ID. NOs. 1, 2 and 3 or the combination of SEQ. ID. NOs. 1, 2, and 4 give a further improved result. The preliminary results indicate that an accuracy of about 90 % was reached using combination of SEQ. ID. NOs. 1, 2, 3 and 4. It was shown that the use of the complete set of SEQ. ID. NOs. 1 through 7 resulted in an accuracy of more than 90 %.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

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Claims

1. A method for the differentiation between ulcerative colitis and Crohn's disease based on the analysis of gene expression profiles in biopsy samples obtained from inflamed and optionally also non-inflamed areas in the intestines of a patient, **characterized** in that the expression levels of at least two of a number of marker genes are determined, said at least two marker genes chosen among SEQ.ID.NO. 1, SEQ.ID.NO. 2, SEQ.ID.NO. 3, SEQ.ID.NO. 4, SEQ.ID.NO. 5, SEQ.ID.NO. 6, and SEQ.ID.NO. 7.

2. The method according to claim 1, wherein the expression levels of SEQ.ID.NO. 1 and SEQ.ID.NO. 2 are determined and wherein the expression of SEQ.ID.NO. 1 in inflamed tissue and optionally the lack of expression in non-inflamed tissue, together with the lack of expression of SEQ.ID.NO. 2 in inflamed tissue or optionally preferential expression in non-inflamed tissue, is taken as an indication of ulcerative colitis.

3. The method according to claim 1, wherein the expression levels of SEQ.ID.NO. 1, SEQ.ID.NO. 2 and SEQ.ID.NO. 3 are determined, and wherein the expression of SEQ.ID.NO. 1 in inflamed tissue and optionally the lack of expression in non-inflamed tissue, together with the lack of expression of SEQ.ID.NO. 2 in inflamed tissue, or optionally preferential expression in non-inflamed tissue, and the expression of SEQ. ID.NO. 3 in inflamed tissue and optionally the lack of expression in non-inflamed tissue, is taken as an indication of ulcerative colitis.

4. The method according to claim 1, wherein the expression levels of SEQ.ID.NO. 1, SEQ.ID.NO. 2 and SEQ.ID.NO. 4 are determined, and wherein the expression of SEQ.ID.NO. 1 in inflamed tissue and optionally the lack of expression in non-inflamed tissue, together with the lack of expression of SEQ.ID.NO. 2 in inflamed tissue, or optionally preferential expression in non-inflamed tissue, and the expression of SEQ. ID.NO. 4 in inflamed tissue and optionally the lack of expression in non-inflamed tissue, is taken as an indication of ulcerative colitis.

5. The method according to claim 1, wherein the expression levels of SEQ.ID.NO. 1 through 7 are determined, and wherein the expression of SEQ.ID.NO. 1, 3, 4, 5, 6, and 7 in inflamed tissue or preferential expression in inflamed tissue, and optionally the lack of expression in non-inflamed tissue, together with the lack of expression of

SEQ.ID.NO. 2 in inflamed tissue, or optionally preferential expression in non-inflamed tissue, is taken as an indication of ulcerative colitis.

6. The method according to any one of the claims above, wherein the expression level of each marker gene is determined through nucleic acid amplification of said genes using gene specific primers, and determination of the amplification results.

7. The method according to claim 6, wherein the nucleic acid amplification is performed using PCR and the gene specific primers chosen among SEQ.ID.NO. 13 – 26.

8. The method according to claim 6, wherein the determination of the amplification results is performed using ethidium bromide staining and visualisation under UV light.

9. A kit for the differentiation between ulcerative colitis and Crohn's disease based on the analysis of gene expression profiles in biopsy samples obtained from inflamed and optionally also non-inflamed areas in the intestines of a patient, **characterized** in that said kit includes gene specific primer pairs directed to at least two marker genes chosen among SEQ.ID.NO. 1, SEQ.ID.NO. 2, SEQ.ID.NO. 3, SEQ.ID.NO. 4, SEQ.ID.NO. 5, SEQ.ID.NO. 6, and SEQ.ID.NO. 7.

10. The kit according to claim 9, wherein said gene specific primer pairs are chosen among SEQ.ID.NO. 13 and SEQ.ID.NO. 14; SEQ.ID.NO. 15 and SEQ.ID.NO. 16; SEQ.ID.NO. 17 and SEQ.ID.NO. 18; SEQ.ID.NO. 19 and SEQ.ID.NO. 20; SEQ.ID.NO. 21 and SEQ.ID.NO. 22; SEQ.ID.NO. 23 and SEQ.ID.NO. 24; and SEQ.ID.NO. 25 and SEQ.ID.NO. 26 or functionally equivalent primer pairs specific for the same marker genes.

11. The kit according to claim 9, wherein said specific primers are SEQ.ID.NO. 13 and SEQ.ID.NO. 14; SEQ.ID.NO. 15 and SEQ.ID.NO. 16; and SEQ.ID.NO. 17 and SEQ.ID.NO. 18.

12. The kit according to claim 9, wherein said specific primers are SEQ.ID.NO. 13 and SEQ.ID.NO. 14; SEQ.ID.NO. 15 and SEQ.ID.NO. 16; and SEQ.ID.NO. 19 and SEQ.ID.NO. 20.

13. The kit according to any one of claims 9 - 12, further comprising a thermostable polymerisation agent and requisite cofactor(-s).

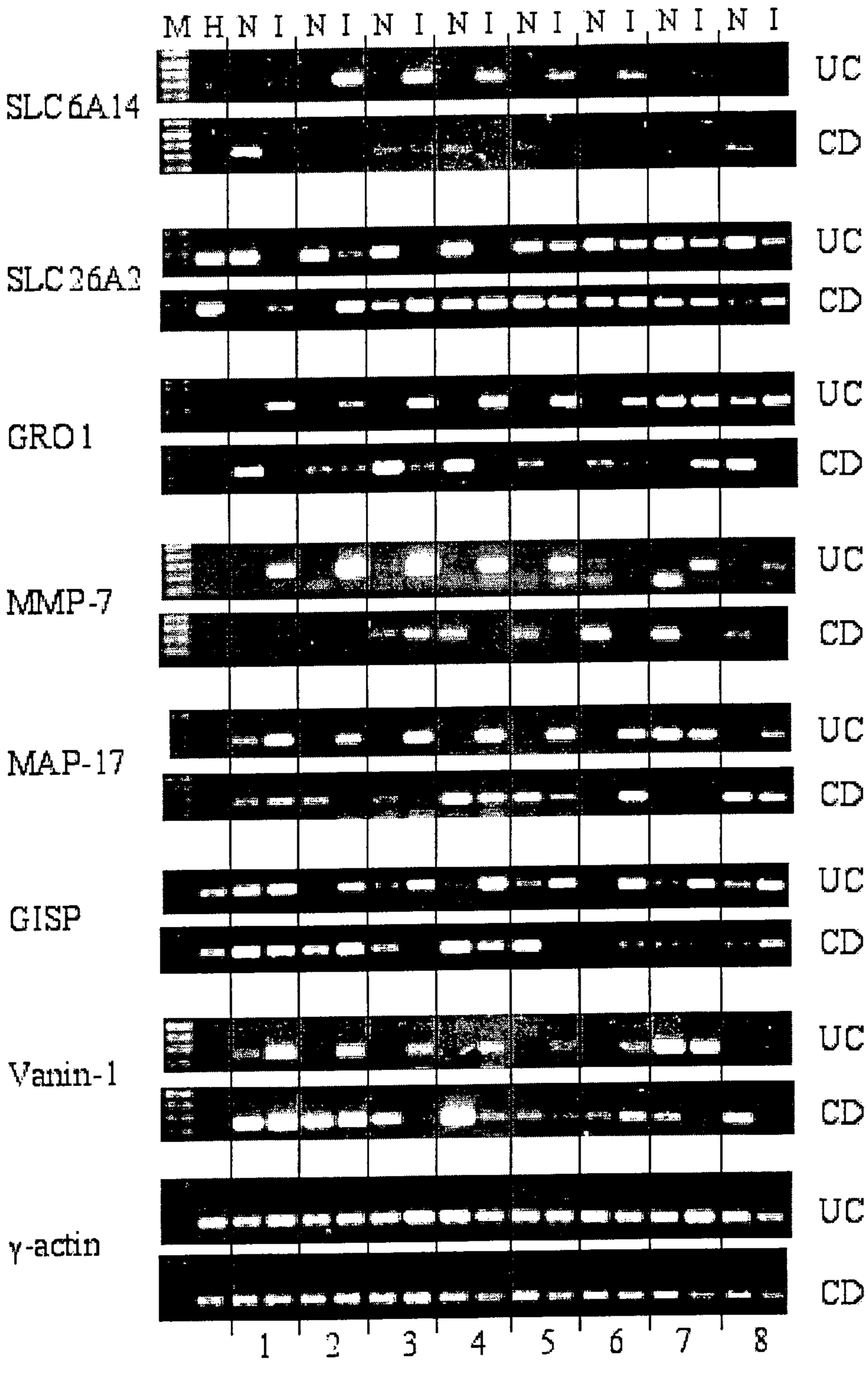
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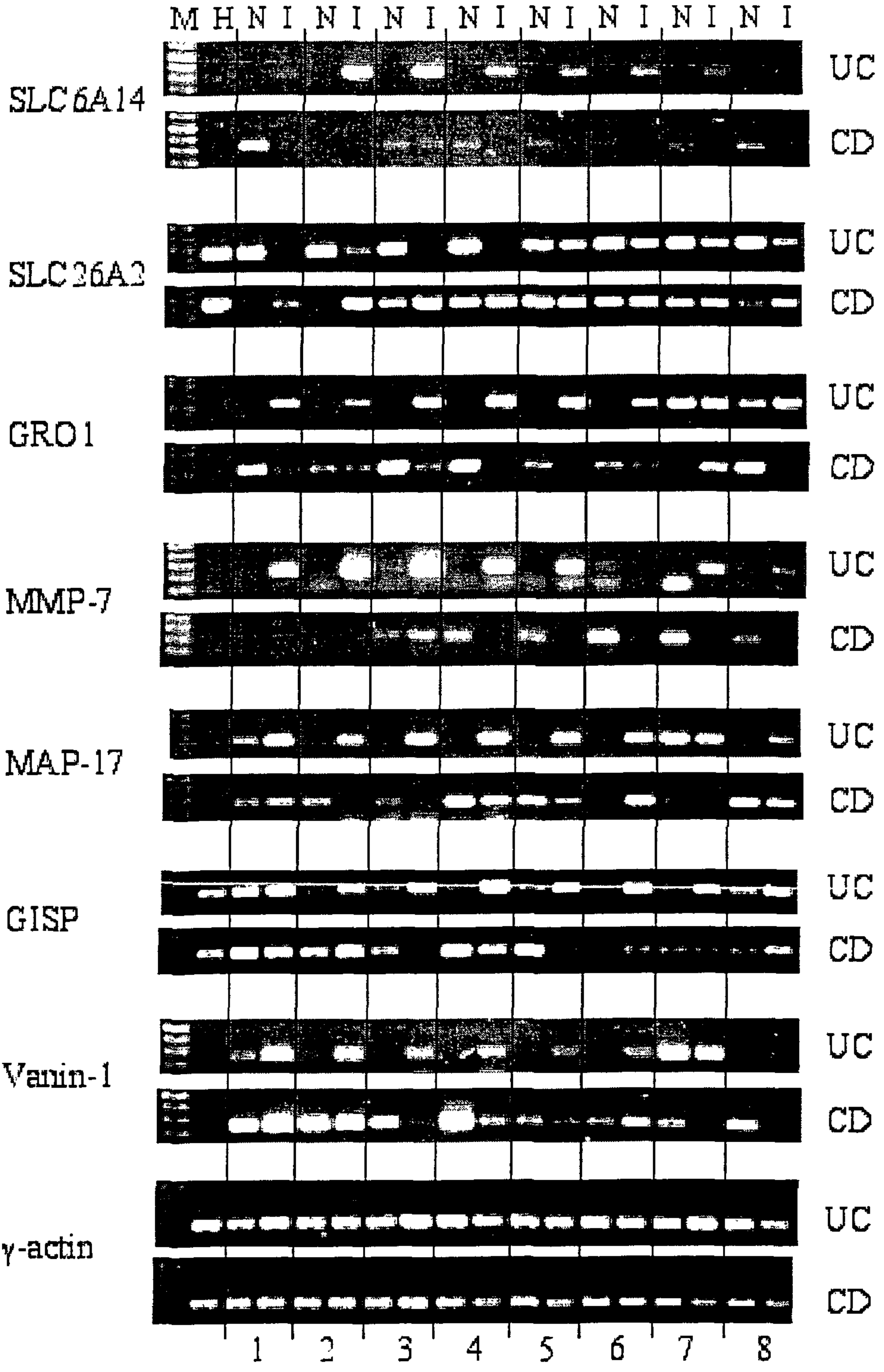
Fig. 1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