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(71) Applicant: **Genox Research, Inc.**
Ibaraki 300-2635 (JP)

(72) Inventors:
• **OHTANI, Noriko,**
Pharmaceutical Research Laboratory
Gunma 370-1295 (JP)

- **MATSUI, Keiko, Tokumajyutaku 433, 1594
Nagano 381-0041 (JP)**
- **YOSHIDA, Nei, c/o Medicinal Genomix Inc.
Yokohama-shi Kanagawa 230-0046 (JP)**
- **SUGITA, Yuji**
c/o Yamanouchi Pharmaceutical Co.Ltd.
Ibaraki 305-8585 (JP)
- **HAMASAKI, Yuhei, c/o Department of Pediatrics
Saga-shi, Saga 849-8501 (JP)**
- **IZUHARA, Kenji, Dept. of Biomolecular Sciences
Saga 849-8501 (JP)**

(74) Representative: **Warcoin, Jacques et al**
Cabinet Régimbeau,
20, rue de Chazelles
75847 Paris Cedex 17 (FR)

(54) **METHOD OF TESTING FOR BRONCHIAL ASTHMA**

(57) SCCA1 and SCCA2 genes, whose expressions were greatly changed in a plurality of cells by stimulating respiratory tract epithelial cells with IL-4 or IL-13, were shown to be allergy related genes. Furthermore, an elevation of expression products of these genes in blood

accompanying bronchial asthmatic attack was elucidated. This invention provides a method of testing for bronchial asthmatic attack that uses as indicators, expression levels of these genes in biological samples.

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DescriptionTechnical Field

5 [0001] The present invention relates to a method of testing for bronchial asthmatic attack.

Background Art

10 [0002] Bronchial asthma is considered to be a multifactorial disease. In other words, bronchial asthma is caused by the interaction of many different genes, each of which is influenced by various environmental factors. Thus, it has been extremely difficult to identify a specific gene that causes bronchial asthma.

[0003] Currently, bronchial asthma is categorized as a chronic inflammatory disease of the respiratory tract. It has been pointed out that allergic reactions at the respiratory tract mucosa and bronchial smooth muscle are closely involved in pathologic formation of bronchial asthma. Therefore, understanding the condition of allergic reactions in these tissues is an important issue in diagnosis of bronchial asthma. In addition, control of allergic reactions is an issue in treatment of bronchial asthma.

20 [0004] On the other hand, the expression of mutated or defective genes, or overexpression or reduction in the expression of specific genes is thought to be involved in allergic diseases. To elucidate the role of gene expression in diseases, it is necessary to understand how a gene is involved in triggering disease onset and how expression of the gene is altered by external stimulants such as drugs.

[0005] Incidentally, atopic diathesis that is accompanied by hyperproduction of IgE antibodies is seen in many bronchial asthma patients. Many causes are considered for bronchial asthma, but there is no doubt that atopic diathesis is a cause of hypersensitivity in many patients. It has been predicted that the mechanism of respiratory tract occlusion in asthmatic attack involves contraction of the bronchial smooth muscle, or edema and respiratory tract endocrine enhancement of the respiratory tract mucosa. I-type allergic reaction in the respiratory tract due to exposure to pathogenic allergens has an important role in such changes in the respiratory tract.

25 [0006] In recent years, IL-4 and IL-13 have been suggested to play important roles in the onset of bronchial asthma. Therefore, for example, in the respiratory tract epithelial cells and bronchial smooth muscles, genes that change their expression level due to IL-4 and IL-13 are thought to be related to bronchial asthma. However, based on this view, there have been no reports on the isolation of genes that specifically change their expression level due to IL-4 and IL-13.

30 [0007] In recent diagnosis of allergic diseases, history taking, and confirmation of the patient's family history and own anamnesis are important factors in general. In addition, for diagnosis of allergy based on more objective information, a test method using patient's blood sample and method for observing patient's immune response to an allergen are also performed. Examples of the former method are the allergen-specific IgE measurement, leukocyte histamine release test, lymphocyte stimulating test, etc. Presence of an allergen-specific IgE is proof for the allergic reaction to that specific allergen. However, in some patients, allergen-specific IgE may not be necessarily detected. Furthermore, the assay principle of IgE requires performing tests for all of the allergens necessary for diagnosis. Leukocyte histamine release test and lymphocyte stimulating test are the methods for observing the immune system reaction toward a specific allergen *in vitro*. These methods are complicated in operation.

35 [0008] On the other hand, another method is also known wherein the immune response observed when a patient is actually contacted with an allergen is used for diagnosing an allergy (latter method). Such a test includes the prick test, scratch test, patch test, intradermal reaction, or induction test. Indeed, these tests allow the direct diagnosis of a patient's allergic reaction; however, they are highly invasive since patients are actually exposed to the allergen.

40 [0009] In addition, regardless of the allergen types, test methods for proving the involvement of allergic reaction are also attempted. For example, a high serum IgE titer may indicate the occurrence of allergic reaction in the patient. The serum IgE titer is information corresponding to the total amount of allergen-specific IgE. Though it is easy to determine the total amount of IgE regardless of the type of allergen, IgE titer may be reduced in some patients with non-atopic bronchitis, etc.

45 [0010] Therefore, a marker (indicator) for an allergic disease that is not only less risky to patients but also capable of readily providing information necessary for diagnosis would be useful.

[0011] Particularly, bronchial asthmatic attack due to allergic reaction markedly inhibits a patient's respiration, and in severe cases, it may bring about death due to respiratory insufficiency. Therefore, prompt identification of the cause and appropriate treatment must be given to patients affected with bronchial asthmatic attack. However, there are no reports on genes found to have a relation to bronchial asthmatic attack.

50 [0012] Regardless of the presence or absence of an attack, there are many reports on attempts to isolate genes relating to patients with allergic diseases. Genes that can be isolated by such an approach can be said to be genes relating to allergic diathesis. In contrast, genes relating to the attack can be expected to be useful as an indicator for diagnosis of the attack and for treatment of attacks needing urgent clinical treatment.

Disclosure of the Invention

[0013] An objective of the present invention is to provide an indicator enabling the test for bronchial asthmatic attack. Another objective of the invention is to provide a method of testing for bronchial asthmatic attack based on the indicator.

[0014] A close involvement of IL-4 and IL-13 in allergic reactions has been suggested by several reports. For example, in an IL-4 knockout mouse (Yssel, H and Groux, H : *Int. Arch. Allergy Immunol.*, 121; 10-18, 2000) and in a STAT6 knockout mouse (Akimoto, T. et al. : *J. Exp. Med.*, 187, 1537-1542, 1998), respiratory tract hypersensitivity disappears. In a mouse model, IL-13 is involved in forming asthma-like pathology regardless of IgE production and Th2 type (Wills-Karp, M. et al.: *Science*, 282, 2258-2261, 1998; Grunig, G. et al.: *Science*, 282, 2261-2263, 1998; Zhu, Z. et al. : *J. Clin. Invest.*, 103, 779-788, 1999).

[0015] Additionally, IL-4 receptors and IL-13 receptors are highly expressed in human respiratory tract epithelial cells and bronchial smooth muscles (Heinzmann, A. et al.: *Hum. Mol. Genet.*, 9: 549-559, 2000). Accordingly, these tissues are thought to be target cells of IL-4 and IL-13. On the other hand, SNP present in IL-4 receptor α and IL-13 were shown to be one of the genetic causes of allergic diseases (Mitsuyasu, H., et al.: *Nature Genet.*, 19, 119-120, 1998; Mitsuyasu, H., et al. : *J. Immunol.*, 162: 1227-1231, 1999; Kruse, S., et al.: *Immunol.*, 96, 365-371, 1999; Heinzmann, A. et al.: *Hum. Mol. Genet.*, 9: 549-559, 2000). Furthermore, inhibition of IL-4 or IL-13 function by soluble IL-4 receptor α was shown to be an effective treatment for bronchial asthma (Borish, L. C. et al.: *Am. J. Respir. Crit. Care Med.*, 160: 912-922, 1999).

[0016] According to the above, a strong relationship to allergic reactions, respiratory symptoms in particular, has been suggested for IL-4 and IL-13. That is, genes constituting signal transduction pathway due to IL-4 and IL-13 may be genes that are closely related to allergic reactions.

[0017] Based on such a line of thought, the inventors thought that if genes indicating changes in expression levels were searched following treatment of human bronchial epithelial cells with IL-4 and IL-13, genes relating to allergic reactions could be isolated. There is a report that attempted a similar approach to isolate genes whose expression level changed following IL-4 and IL-13 treatment (Wang et al., *Immunology* 2000, Seattle, May 12-16, 2000). However, since in known searching methods, the number of lots of cells used for analysis is small, and the range of changes in expression levels are not clear, specificity towards stimulation from IL-4 and IL-13 cannot be expected.

[0018] Therefore, to isolate genes that respond with greater specificity towards IL-4 and IL-13 stimulation, the inventors increased the number of lots of cells that became the object of analysis, and furthermore, selected those in which the change in expression level reached twice as much or more. Next, following stimulation with IL-4 and IL-13 of the respiratory tract epithelial cells, a significant elevation in the expression level of genes selected this way was confirmed.

[0019] Next, to confirm the relationship of selected genes to bronchial asthma, levels of each gene product in the serum of bronchial asthma patients under various conditions were measured. As a result, specific gene products were found to show significantly higher values in the serum of bronchial asthma patients who have had attacks. Based on such findings, the inventors elucidated the existence of SCCA1 and SCCA2 genes having a close relationship to bronchial asthmatic attacks.

[0020] Based on the above findings, the inventors found that tests for bronchial asthmatic attacks become possible by using these genes and proteins encoded by these genes as indicators, and completed this invention.

[0021] That is, this invention relates to a testing method described below, and its reagents.

(1) A method of testing for bronchial asthmatic attack using SCCA1 and/or SCCA2 as an indicator gene, said method comprising the steps of:

- (a) measuring the expression level of the indicator gene in a biological sample from a subject, and
- (b) comparing the expression level measured in (a) with that in a biological sample from a living body not affected by bronchial asthma.

(2) The method of (1), wherein the biological sample is blood.

(3) The method of (1), wherein the expression level of the gene is measured by PCR of the cDNA for the gene.

(4) The method of (1), wherein the expression level of the gene is measured by detecting a protein encoded by the gene.

(5) A reagent for testing for bronchial asthmatic attack, said reagent comprising an oligonucleotide that is at least 15 nucleotides long and that has a nucleotide sequence complementary to a polynucleotide having a nucleotide sequence of the SCCA1 and/or SCCA2 gene or to a complementary strand thereof.

(6) A reagent for testing for bronchial asthmatic attack, said reagent comprising an antibody recognizing a peptide having an amino acid sequence of SCCA1 and/or SCCA2 protein.

[0022] Incidentally, the existence of both SCCA1 and SCCA2 genes has been elucidated. Both SCCA1 and SCCA2

have been reported as genes encoding squamous cell carcinoma antigens, and are 45 kD genes belonging to the OVA family. They show 98% homology in the nucleotide sequences in the exon and show 92% homology at the amino acid level. The differences in the two nucleotide sequences can be discerned by PCR, etc. In the genome, both genes are mapped in 18q21.3 (Schneider, S. S. et al., Proc. Natl. Acad. Sci. USA, 92, 3147-3151, 1995). SCCA1 and SCCA2

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SCCA1; squamous cell carcinoma antigen 1 (cysteine protease inhibitor):

used as a diagnostic marker for squamous cell carcinoma (DE4139418-A).

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SCCA2; squamous cell carcinoma antigen 2 (serine protease inhibitor):

besides diagnosis marker, it is applied to the regulation of cell growth and apoptosis, or its agonist is applied to the treatment of cancer or psoriasis (WO9714425-A1).

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[0023] Furthermore, it has been elucidated that in response to IL-4 and IL-13, expression of both of these genes is enhanced in the respiratory tract epithelial cells (Wang et al., Immunology 2000, Seattle, May 12-16, 2000). However, it was unknown that the blood level of the protein encoded by these genes is significantly elevated with bronchial asthmatic attack.

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[0024] In this invention, bronchial asthmatic attack refers to a condition where respiratory tract stenosis occurs due to allergic reactions in the respiratory tract. More specifically, bronchial asthmatic attack is caused by contraction of the respiratory tract smooth muscles and by mucus in the bronchus and bronchiolar air cavity. Such changes in the respiratory tract are thought to be induced by smooth muscle contraction effect and vasostimulant effect of histamine, leukotriene, or prostaglandin, etc.

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[0025] The method of testing for bronchial asthmatic attack of this invention includes the steps of measuring the expression level of SCCA1 and/or SCCA2 genes in a biological sample from a subject, and comparing the measured value with that for a living body not affected by bronchial asthma. As a result of comparing the two, when expression is enhanced compared to the living body not affected by bronchial asthma, the subject is judged to be in a condition of being affected with bronchial asthmatic attack. In this invention, a living body not affected by bronchial asthma includes healthy subjects and living body that is not exactly healthy but that has a condition clearly diagnosed not as bronchial asthma.

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[0026] In this invention, SCCA1 and SCCA2 genes together, which can be indicators of bronchial asthmatic attack, are referred to as indicator genes. In this invention, SCCA1 and SCCA2 include not only those of human origin but also homologues of other species. Therefore, indicator genes in species other than humans, unless otherwise stated, refer to homologues of SCCA1 and SCCA2 genes intrinsic to those species, or either one of exogenous SCCA1 gene or SCCA2 gene that has been transformed into that individual.

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[0027] In the blood of patients affected with bronchial asthmatic attack, the measured value of the indicator protein of this invention showed a significant increase. Therefore, testing for a bronchial asthmatic attack can be performed using the expression level of these indicator genes.

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[0028] In this invention, the expression level of indicator genes includes transcription of these genes to mRNA, and translation into proteins. Therefore, the method of testing for bronchial asthmatic attack of this invention is performed based on the expression intensity of mRNA corresponding to the aforementioned genes, or on the comparison of expression level of proteins encoded by the aforementioned genes.

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[0029] Usually, to compare the expression levels, a standard value is set based on, for example, the expression level of the aforementioned indicator genes in healthy subjects. Based on this standard value, a range of ± 2 S.D. for example, is set as the permitted limit. The technique of setting standard values and permitted limits based on the measured value of indicator genes is well known. If the expression level of indicator genes in a subject is higher than the permitted limit, that subject is considered susceptible to a bronchial asthmatic attack. Additionally, if the expression level is within the permitted limit, or exceeds the permitted limit, the possibility of having had a bronchial asthmatic attack is predicted to be low.

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[0030] Measurement of the expression level of an indicator gene in a test for bronchial asthmatic attack in the present invention can be performed according to the known gene analytical method. More specifically, for example, a hybridization technique with a nucleic acid as a probe that hybridizes to this gene, a gene amplification technique with a DNA hybridizing to the gene of this invention as a primer, etc. can be utilized.

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[0031] The probe or primer used in the test of the present invention can be designed based on the nucleotide sequence of the indicator gene. The nucleotide sequence of the indicator gene is known. GenBank accession No. corresponding to the nucleotide sequence of each indicator gene is shown in Examples.

[0032] Genes of higher animals are generally accompanied by polymorphism in a high frequency. Many molecules produce isoforms comprising different amino acid sequences from each other during the splicing process. Any genes

associated with allergy that have a similar activity to that of the indicator gene are included in the indicator gene of the present invention, even though they carry mutation in the nucleotide sequence due to polymorphism and isoform.

[0033] A primer or probe can be a polynucleotide comprising the nucleotide sequence of the indicator gene or at least 15 nucleotides that are complementary to the complementary strand thereof. Herein, the term "complementary strand" means one strand of a double stranded DNA composed of A: T (U for RNA) and G: C base pairs, when viewed against the other strand. In addition, "complementary" means not only those completely complementary to a region of at least 15 continuous nucleotides, but also having a homology of at least 70%, preferably at least 80%, more preferably 90%, and even more preferably 95% or higher. The degree of homology between nucleotide sequences can be determined by the algorithm, BLAST, etc.

[0034] Such polynucleotides are useful as probes to detect an indicator gene, or as a primer to amplify the indicator gene. When used as a primer, these polynucleotides usually comprise 15 bp~ 100 bp, preferably 15 bp~ 35 bp of nucleotides. When used as a probe, DNAs comprise the whole sequence of the indicator gene (or a complementary strand thereof), or a partial sequence thereof contains at least 15-bp nucleotides. When used as a primer, the 3' region thereof must be complementary to the indicator gene, while the 5' region can be linked to a restriction enzyme-recognition sequence or tag.

[0035] "Polynucleotides" in the present invention may be either DNA or RNA. These polynucleotides may be either synthetic or naturally-occurring. Also, DNA used as a probe for hybridization is usually labeled. Examples of labeling methods are those as described below. Herein, the term "oligonucleotide" means a polynucleotide with relatively low degree of polymerization. Oligonucleotides are included in polynucleotides. The labeling methods are as follows:

- nick translation labeling using DNA polymerase I;
- end labeling using polynucleotide kinase;
- fill-in end labeling using Klenow fragment (Berger, SL, Kimmel, AR. (1987) Guide to Molecular Cloning Techniques, Method in Enzymology, Academic Press; Hames, BD, Higgins, SJ (1985) Genes Probes: A Practical Approach. IRL Press; Sambrook, J, Fritsch, EF, Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press);
- transcription labeling using RNA polymerase (Melton, DA, Krieg, PA, Rebagkiati, MR, Maniatis, T, Zinn, K, Green, MR. (1984) Nucleic Acid Res., 12, 7035-7056); and
- non-isotopic labeling of DNA by incorporating modified nucleotides (Kricka, LJ. (1992) Nonisotopic DNA Probing Techniques. Academic Press).

[0036] For testing for bronchial asthmatic attack using hybridization techniques, for example, Northern hybridization, dot blot hybridization, or DNA microarray technique may be used. Furthermore, gene amplification techniques, such as RT-PCR method may be used. By using the PCR amplification monitoring method during the gene amplification step in RT-PCR, one can achieve more quantitative analysis for the gene expression of the present invention.

[0037] In the PCR gene amplification monitoring method, the detection target (DNA or reverse transcript of RNA) is hybridized to probes that are dual-labeled at both ends with different fluorescent dyes whose fluorescence cancel each other out. When the PCR proceeds and Taq polymerase degrades the probe with its 5' -3' exonuclease activity, the two fluorescent dyes become distant from each other and the fluorescence becomes to be detected. The fluorescence is detected in real time. By simultaneously measuring a standard sample in which the copy number of the target is known, it is possible to determine the copy number of the target in the subject sample with the cycle number where PCR amplification is linear (Holland, P. M. et al., 1991, Proc. Natl. Acad. Sci. USA 88: 7276-7280; Livak, K. J. et al., 1995, PCR Methods and Applications 4 (6) : 357-362; Heid, C. A. et al., 1996, Genome Research 6: 986-994; Gibson, E. M. U. et al., 1996, Genome Research 6: 995-1001). For the PCR amplification monitoring method, for example, ABI PRISM7700 (PE Biosystems) may be used.

[0038] The method of testing for bronchial asthmatic attack in the present invention can be also carried out by detecting a protein encoded by the indicator gene. Hereinafter, a protein encoded by the indicator gene is described as an indicator protein. For such test methods, for example, Western blotting method, immunoprecipitation method, and ELISA method may be employed using antibody that binds to the indicator protein.

[0039] Antibodies that bind to the indicator protein used in the detection may be produced by techniques known to those skilled in the art. Antibodies used in the present invention may be polyclonal or monoclonal antibodies (Milstein, C. et al., 1983, Nature 305 (5934): 537-40). For example, polyclonal antibody against an indicator protein may be produced by collecting the blood from mammals sensitized with the antigen, and separating the serum from this blood using known methods. As a polyclonal antibody, the serum containing polyclonal antibody as such may be used. As the occasion demands, a fraction containing polyclonal antibody can be further isolated from this serum. Also, monoclonal antibody may be obtained by isolating immune cells from mammals sensitized with the antigen, fusing these cells with myeloma cells, etc. cloning hybridomas thus obtained, and collecting the antibody as a monoclonal antibody from the culture of the hybridomas.

[0040] For detecting an indicator protein, these antibodies may be appropriately labeled. Alternatively, instead of labeling the antibody, a substance that specifically binds to the antibody such as protein A or protein G, may be labeled to arrange an indirect detection of indicator protein. More specifically, one example of an indirect detection method is ELISA.

5 [0041] Protein or its partial peptide used as an antigen may be obtained, for example, by inserting the gene or its portion into an expression vector, introducing it into an appropriate host cell to produce a transformant, culturing the transformant to express the recombinant protein, and purifying the expressed recombinant protein from the culture or the culture supernatant. Alternatively, amino acid sequences encoded by these genes, or oligopeptides comprising portions of the amino acid sequence encoded by the full-length cDNA are chemically synthesized to be used as the antigen.

10 [0042] In the method of testing for bronchial asthmatic attack based on this invention, the indicator genes that become the object of expression level and activity measurements are SCCA1 and/or SCCA2. SCCA1 and SCCA2 have very similar structures and it is difficult to differentiate the two immunologically. For example, a kit to measure SCCA proteins immunologically is commercially available; however, the measured value of SCCA obtained using this kit is the sum of SCCA1 and SCCA2 values. That is, at present, measurements differentiating the two are not possible with the commercially available kit.

15 [0043] However, the expression level of both of the indicator genes of this invention was found to rise accompanying bronchial asthmatic attack. Therefore, bronchial asthmatic attack can be tested by measuring the overall expression level of these indicator genes.

20 [0044] Incidentally, monoclonal antibodies capable of differentiating the two have been reported. Therefore, it is possible to selectively measure the two by immunological techniques (Clin. Chim. Acta 2000 295 107-127).

[0045] Furthermore, in this invention, bronchial asthmatic attack can be tested not only by the expression level of indicator genes, but also by using the activity of indicator proteins in biological samples as an indicator. The activity of indicator proteins refers to biological activity equipped in each protein. Detection of activity of the aforementioned indicator proteins can be performed based on known methods. Methods for measuring the activity of SCCA1 and SCCA2 are well known.

25 [0046] First, in the case of SCCA1, its activity can be measured as cysteine protease inhibitor activity of cathepsin-K, L, or S (Biochemistry 1998 37 5258-5266). On the other hand, SCCA2 has serine protease inhibitor activity of cathepsin-G and chymase. Therefore, the activity of SCCA2 can be measured as inhibitor activity (J.B.C. 1997 272 1849-1855). Specifically, activity of these indicator proteins can be found out by adding SCCA1 or SCCA2 with substrates of various proteases, and by using enzyme activity suppression as an indicator.

30 [0047] Normally, in the testing method of this invention a biological sample collected from a subject is used. Blood sample is preferred as the biological sample. As blood samples, whole blood, or plasma and serum obtained from whole blood may be used. Additionally, as biological samples of this invention, expectoration, nasal mucous secretion, bronchoalveolar lavage fluid, pulmonary abrasion cells, etc. may be used besides blood. Collection methods for these biological samples are well known.

35 [0048] When the biological sample is cells of respiratory tract epithelia, etc., samples for immunological measurements of the aforementioned proteins can be made by preparing a lysate. Otherwise, samples for measuring mRNA corresponding to the aforementioned genes can be made by extracting mRNA from this lysate. For extraction of lysate and mRNA of the biological sample, it is useful to utilize a commercially available kit. Otherwise, biological samples in the liquid form such as blood, nasal mucous secretion, and bronchoalveolar lavage fluid can be made into samples for measurement of proteins and genes by diluting with a buffer, etc., as necessary.

40 [0049] The measured value of expression level of indicator genes in cells can be corrected by known methods. As a result of correction, change in gene expression level in cells can be compared. Based on the measured value of expression level of genes that are expressed in the respiratory tract epithelial cells and do not show large fluctuations in their expression level regardless of the condition of the cell (housekeeping genes), correction of the measured value is performed by correcting the measured value of expression level of the genes that are to be used as indicators in this invention.

45 [0050] Furthermore, the present invention provides a reagent for the testing method of this invention. That is, this invention relates to a reagent for testing for bronchial asthma, said reagent comprising an oligonucleotide that comprises a nucleotide sequence complementary to a polynucleotide containing the nucleotide sequence of the indicator gene or to the complementary strand thereof and that comprises at least 15 nucleotides. Alternatively, this invention relates to a reagent for testing for bronchial asthma, said reagent comprising an antibody that recognizes a peptide containing the amino acid sequence of the indicator protein. Oligonucleotides and antibodies composing the reagent of this invention may be appropriately labeled, or immobilized onto a suitable carrier according to the assay format. Further, the reagent of this invention may be combined with, as well as the oligonucleotides or antibodies as described above, additional elements necessary for the test or storage to form a kit. Additional elements that can be used for constituting a kit are shown below. These elements may be previously mixed as necessary, or added with preservatives

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and antiseptics:

buffer for diluting reagents and biological samples;
 positive standard sample;
 5 negative standard sample;
 substrate for measuring labels;
 reaction vessel; and
 manual describing the assay protocol.

10 **[0051]** Tests of bronchial asthmatic attack in this invention include, for example, the following tests. Even for patients who show bronchial asthmatic symptoms yet cannot be judged to have allergic diseases by general testing, by performing the test based on this invention, one can easily judge that the patients are in a condition where bronchial asthmatic attack due to allergic reactions occurs.

15 **[0052]** More specifically, elevation of indicator gene expression in patients indicating asthmatic attack indicates high probability that the cause of that symptom is an allergic disease. Among bronchial asthmatic attacks, there are those caused by allergic reactions and others that are not. The treatment methods for the two are completely different. Therefore, to diagnose by which cause the bronchial asthmatic symptoms are being manifested is an extremely important step in treatment. The testing method of this invention provides extremely important information in identifying the cause of bronchial asthma.

20 **[0053]** Otherwise, this invention enables testing to judge whether the bronchial asthmatic attack is improving or not. The indicator proteins of this invention showed significant elevation of the measured value in the blood of patients affected with bronchial asthmatic attack. In addition, the indicator genes of this invention are genes that increase their expression in respiratory tract epithelial cells stimulated with IL-4 or IL-13. Respiratory tract epithelial tissues are tissues that show remarkable lesions in bronchial asthma. Therefore, proteins that are expression products of genes with
 25 fluctuating expression in respiratory tract epithelial cells stimulated with IL-4 or IL-13, which are cytokines that strongly induce allergic reactions, and those in which their measured value fluctuates in the blood are useful for judgment of therapeutic effect. More specifically, elevation of the measured value of indicator proteins in patients diagnosed with bronchial asthmatic attack indicates strong possibility that the attack is aggravated.

30 **[0054]** Tests of this invention include tests for predicting the possibility that bronchial asthmatic attack may occur in a relatively short time after testing, or for finding out, after recovery from a respiratory disorder, whether the cause of that respiratory disorder was due to bronchial asthma. Prediction of the attack is important in securing safety for the subjects. Additionally, finding out the cause of respiratory disorders experienced previously by the subject becomes important information in determining a therapeutic course for a patient.

35 Brief Description of the Drawings

[0055]

40 Fig. 1 is a set of graphs indicating the result of measuring the expression level (copy/5 ng RNA) of SCCA1 gene in cultivated bronchial epithelial cells stimulated with IL-4 and IL-13, or with other cytokines. The top left is a graph indicating changes in expression level with time, 0, 6, 12, 24, and 48 hours after treatment. The numbers on the abscissa show the cultivation time. The top right is a graph indicating the change in expression level 24 hours after treatment with other cytokines. The bottom left is a graph indicating the expression level in each lot 24 hours after IL-4 and IL-13 stimulation. The bottom right is a graph indicating the changes in expression level 0, 24, 48, 72,
 45 and 96 hours after treating BEAS-2B cells with IL-4 and IL-13 stimulation. From left to right, each of the columns indicates actual measurement (raw data), β -actin scale factor (β -actin), and GAPDH scale factor (GAPDH).

Fig. 2 is a set of graphs indicating the result of measuring the expression level of SCCA2 gene in cultivated bronchial epithelial cells simulated with IL-4 and IL-13, or with other cytokines. Each graph indicates analogous content to that of each graph in Figure 1.

50 Fig. 3 is a graph indicating the measured value of serum SCCA in bronchial asthma patients and in non-asthma patients.

Fig. 4 is a graph indicating the measured value of serum SCCA in the same asthma patient during attack and during absence of an attack.

55 Best Mode for Carrying out the Invention

[0056] The present invention will be explained in detail below with reference to examples, but it is not to be construed as being limited thereto.

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[Example 1] Selection of candidate genes using DNA microarray

1. Cultivation of normal human bronchial epithelial cells, and IL-4 or IL-13 stimulation

5 **[0057]** Three lots of normal human bronchial epithelial cells available from Clonetics were purchased (8F1756, 8F1548, 8F1805). Cells (5×10^5) contained in one vial were divided into three equal parts ($1.67 \times 10^5/75$ cm² flask) for no stimulation, IL-4 stimulation, and IL-13 stimulation, and these were cultivated for approximately 8 to 10 days in SABM media (Clonetics) with medium exchange. During this procedure, BPE (bovine pituitary extract), hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, BSA-FAF, triiodothyronine, GA-1000 (gentamicin/amphotericin-B) were added to the media according to the attached protocol.

10 **[0058]** Before cytokine stimulation, the cells were washed with PBS, and then placed into SABM without added factors. IL-4 (10 ng/mL) or IL-13 (50 ng/mL) (both from Peprotech) were added thereto, and this was cultivated for 24 hours. Observation of changes with passage of time (0, 6, 12, 24, and 48 hours) was carried out in a similar manner.

15 2. Other cytokine stimulation of normal human bronchial epithelial cells

[0059] Using cells from lot 8F1548, cultivation was performed similarly to that of 1. In place of IL-4 or IL-13, 50 ng/mL of TNF α , IL-1 β , IL-5, IL-6, or IL-9 (all from Peprotech) were added and cultivated for 24 hours.

20 3. Preparation of RNA for GeneChip

[0060] Respiratory tract epithelial cells treated as mentioned above were dissolved in Isogen (Nippon Gene; Wako Pure Chemicals), and from this solution, RNA was separated according to a protocol attached to Isogen. After addition of chloroform, this was agitated, then centrifuged, and its aqueous layer was collected. Next, isopropanol was added, this was agitated, and then centrifuged to collect the precipitated total RNA.

25 4. cDNA synthesis for GeneChip

[0061] Single stranded cDNA was prepared from 5 μ g of total RNA, which was prepared from cells of lot 8F1756, by reverse transcription using Superscript II Reverse Transcriptase (Life Technologies) following the method of Expression Analysis Technical Manual by Affymetrix, and by using T7- (dT)₂₄ (Amersham Pharmacia) as a primer. The T7- (dT)₂₄ primer comprises a nucleotide sequence in which d(T)₂₄ is added to a T7 promoter nucleotide sequence, as shown below.

35 T7-(dT)₂₄ primer (SEQ ID NO: 1)

5' -GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG- (dT)₂₄-3'

[0062] Next, according to Expression Analysis Technical Manual, DNA ligase, DNA polymerase I, and RNase H were added to synthesize double stranded cDNA. After phenol-chloroform extraction of cDNA, this was passed through Phase Lock Gels, and was purified by ethanol precipitation.

[0063] Furthermore, using BioArray High Yield RNA Transcription Labeling Kit, biotin-labeled cRNA was synthesized. Using RNeasy Spin column (QIAGEN), cRNA was purified and then fragmented by treatment with heat.

45 **[0064]** To a hybridization cocktail, 12.5 μ g of this cRNA was added according to Expression Analysis Technical Manual. This was placed into an array and was hybridized for 16 hours at 45°C.

[0065] After the array was washed, streptavidin phycoerythrin was added for staining. After washing, a mixed antibody solution of normal goat IgG and biotinylated goat IgG was added to the array. Furthermore, in order to enhance fluorescence intensity, streptavidin phycoerythrin was added again for staining. After washing, this was set into a scanner and was analyzed by a GeneChip software.

50 5. GeneChip analysis

[0066] Data analysis was performed using Suite, which is a GeneChip analysis software. Average Intensity (1) and Background Average (2) were investigated by Absolute Analysis, and 3 average values obtained for no stimulation, IL-4 stimulation, and IL-13 stimulation by subtracting (2) from (1) were used as scale factors for comparison analysis.

55 **[0067]** First, absolute analysis was performed to analyze one chip data. Positives and negatives were determined by comparing the fluorescence intensity of perfect match and mismatch of a probe set. Judgment into three categories of Absolute Calls, which are P (present), A (absent), and M (marginal), were made by values of Pos Fraction, Log

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Avg, and Pos/Neg.

Pos Fraction; ratio of positive pairs.

Log Avg; average of the log of fluorescence intensity ratio between probe cells of perfect match and mismatch.

Pos/Neg; ratio of the number of positive pairs and negative pairs.

5 [0068] Additionally, Average Difference (Avg Diff), which is the average value of the difference in fluorescence intensities between probe cells of perfect match and mismatch, was calculated for each gene.

[0069] Next, Comparison Analysis was performed on two sets of data. Comparisons were made between no stimulation and IL-4 stimulation, or between no stimulation and IL-13 stimulation, and differences in expression levels were ranked as follows. Judgment into 5 categories of difference calls, which are I, D, MI, MD, and NC, were made from
10 values of Inc/Dec, Inc Ratio, Dpos-Dneg Ratio, and Log Avg Ratio Change.

Inc: Number of probe pairs that corresponded to IL-4 stimulation or IL-13 stimulation and no stimulation and that were judged to show increased expression levels for IL-4 stimulation or IL-13 stimulation.

Dec: Number of pairs judged to show decreased expression levels for IL-4 stimulation or IL-13 stimulation.

15 Inc/Dec: Ratio of the number of pairs judged to be Inc and number of pairs judged to be Dec.

Inc Ratio: Number of pairs judged to be Inc/number of pairs actually used.

Dpos/Dneg Ratio: Ratio between the number of Neg Change subtracted from that of Pos Change, and the number of pairs actually used.

20 Pos Change: Difference between the number of positive pairs in Absolute Analysis of IL-4 stimulation or IL-13 stimulation, and the number of positive pairs in Absolute Analysis of no stimulation.

Neg Change: Difference between the number of negative pairs in Absolute Analysis of IL-4 stimulation or IL-13 stimulation, and the number of negative pairs in Absolute Analysis of no stimulation.

Log Avg Ratio Change: Difference between Log Avg in Absolute Analysis of IL-4 stimulation or IL-13 stimulation and no stimulation.

25 Increased: I,

Decreased: D,

Marginally Increased: MI,

Marginally Decreased: MD, and

No Change: NC

30 [0070] Additionally, S66896 and U19557 (both are GenBank Acc. No.) were selected as genes whose expression was enhanced due to IL-4 stimulation or IL-13 stimulation by values of Fold Change, which is a ratio of Avg Diff in Absolute Analysis of no stimulation with IL-4 stimulation or no stimulation with IL-13 stimulation. S66896 probe corresponds to SCCA1 gene, and U19557 to SCCA2. Table 1 shows the value of Fold Change of 566896 and U19557 in
35 respiratory tract epithelial cells by lot number.

Table 1

Probe	Gene name	IL-4		
		8F1756	8F1548	8F1805
S66896	SCCA1	22.5	11.1	14.5
U19557	SCCA2	21.7	25.6	19.4
Probe	Gene name	IL-13		
		8F1756	8F1548	8F1805
S66896	SCCA1	17.4	14.1	7.1
U19557	SCCA2	15.6	28.8	18.7

40 [0071] As evident from Table 1, SCCA1 and SCCA2 are genes that show 10- to 20-fold or greater expression level due to stimulation by either IL-4 or IL-13, which are allergy related cytokines and are closely related to allergies. Furthermore, changes in the expression levels of these genes were commonly seen in different lots.

[Example 2] Confirmation of expression level of candidate genes

55 [0072] To quantitatively confirm the expression level of SCCA1 and SCCA2 genes selected in Example 1, cultivated respiratory tract epithelial cells (Clonetics) were used to further perform quantitative PCR by ABI 7700. Three lots, 8F1756, 8F1548, and 8F1805, were used for the cultivated cells. Primer and TaqMan probe used for measurements

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by ABI 7700 were designed by Primer Express (PE Biosystems) based on sequence information of each gene. The 5'-end of TaqMan probe is labeled with FAM (6-carboxy-fluorescein) and the 3'-end is labeled with TAMRA (6-carboxy-N, N,N',N'-tetramethylrhodamine). Nucleotide sequences of oligonucleotides used for forward primer (F) , reverse primer (R) , and TaqMan probe (TP) of each gene are as shown below. GenBank Accession No. corresponding to the nucleotide sequence of each indicator gene is shown in parenthesis following the name.

SCCA1 (s66896)

[0073]

F: agccgcggtctctgtgc (SEQ ID NO: 2)

R: ggcagctgcagcttctg (SEQ ID NO: 3)

TP: aaggcctttgtggagggttacagaggagga (SEQ ID NO: 4)

SCCA2 (u19557)

[0074]

F: agccacggtctctcag (SEQ ID NO: 5)

R: gcagctgcagcttcca (SEQ ID NO: 6)

TP: aaggcctttgtggagggtcactgaggagga (SEQ ID NO: 7)

[0075] Total RNA extracted by the aforementioned method was treated with DNase (Nippon Gene). Then, cDNA, which was reverse transcribed using random hexamer (GIBCO BRL) as primer, was used as a template. For a standard curve to calculate the number of copies, a plasmid clone containing a nucleotide sequence region that is amplified by both primers was prepared for each of the genes, and this was diluted stepwise to be used as template for carrying out the reaction. The composition of reaction solution for monitoring PCR amplification is shown in Table 2.

Table 2

Composition of reaction in ABI-PRISM 7700 (Amount per well)	
Sterilized distilled water	23.75 (μL)
10x TaqMan buffer A	5
25mM MgCl ₂	7
dATP(10 mM)	1.0
dCTP(10 mM)	1.0
dGTP(10 mM)	1.0
dUTP(20 mM)	1.0
Forward Primer (10 μM)	1.0
Reverse Primer (10 μM)	1.0
TaqMan probe (2.0 μM)	2.5
AmpliTaq Gold (5 U/μL)	0.25
AmpErase UNG (1 U/μL)	0.5
Template solution	5
Total	50

[0076] Additionally, to correct the differences of cDNA concentration in the sample, similar quantitative analysis was performed for β -actin gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as internal standards for correction. By correcting based on the number of copies of these genes, the number of copies of the genes of interest was calculated.

[0077] Primers and probes for measuring β -actin or GAPDH were those packaged with TaqMan β -actin Control Reagents (PE Biosystems). The nucleotide sequences are as shown below. The β -actin-corrected expression levels (copy/5 ng RNA) for each of the genes are shown in Figs. 1 and 2.

[0078] Furthermore, the results of averaging fold change values from three lots when β -actin correction was performed and the unstimulated value was taken to be 1, are shown in Table 3.

Table 3

Name of gene	IL-4 stimulation	IL-13 stimulation
SCCA1	30.3±18.7	25.3±10.3
SCCA2	55.9±23.0	56.6±16.1

β -actin forward primer (SEQ ID NO: 8)
TCA CCC ACA CTG TGC CCA TCT ACG A

β -actin reverse primer (SEQ ID NO: 9)
CAG CGG AAC CGC TCA TTG CCA ATG G

β -actin TaqMan probe (SEQ ID NO: 10)
(FAM) ATGCCCT-T (TAMRA) -CCCCCATGCCATCCTGCGTp-3'

GAPDH forward primer (SEQ ID NO: 11)
GAAGGTGAAGGTCGGAGT

GAPDH reverse primer (SEQ ID NO: 12)
GAAGATGGTGATGGGATTTC

GAPDH TaqMan probe (SEQ ID NO: 13)
(FAM) CAAGCTTCCCGTTCTCAGCC (TAMRA) -3'

FAM: 6-carboxy-fluorescein

TAMRA: 6-carboxy-N,N,N',N'-tetramethylrhodamine

[0079] As a result of quantitative PCR, the expression levels of both SCCA1 and SCCA2 genes in the respiratory tract epithelial cells, where both genes were selected in Example 1, were elevated by several tenfold or more in three different respiratory tract epithelial cells due to IL-4 or IL-13 stimulation. Based on these results, elevation of expression levels of these indicator genes in response to IL-4 and IL-13 could be predicted in respiratory tract epithelial cells.

[0080] The indicator genes of this invention show common behavior among different lots of bronchial epithelial cells by IL-4 and IL-13 stimulation known to have close relationship to allergic reactions. Therefore, the indicator genes of this invention can be thought to be important genes regulating the progress of allergic reactions.

[Example 3] Analysis of blood SCCA

[0081] To further confirm the relation of SCCA1 and SCCA2 genes, which were found to be related to allergic reactions in Examples 1 and 2, to bronchial asthmatic attack, blood levels of proteins encoded by these genes were measured

in living bodies under various conditions.

[0082] Serum was separated from blood of bronchial asthma patients and non-asthma patients, or from the same bronchial asthma patient during attack and during absence of the attack. Using an SCCA measurement kit (IMx, Dainabot), blood level of SCCA protein containing both SCCA1 and SCCA2 was measured. SCCA measurement kit uses antibodies that react against both SCCA1 and SCCA2, therefore a total of these indicator proteins can be measured.

[0083] As a result, when bronchial asthma patients and non-asthma patients were compared, the blood SCCA level was significantly elevated in bronchial asthma patients (Fig. 3). Comparing the blood SCCA level in the same asthma patient during attack and during absence of an attack, the level was significantly elevated during attack (Fig. 4).

[0084] From these results, SCCA1 and SCCA2 were found to be genes related to allergies, and simultaneously, it was found that their blood levels significantly elevate during bronchial asthmatic attack compared to a healthy person or compared to a bronchial asthma patient during absence of the attack.

Industrial Applicability

[0085] This invention elucidated genes whose expression increases during bronchial asthmatic attack. In this invention, SCCA1 and SCCA2 genes have been found to be related to bronchial asthma. It has been found in this invention that these genes increase their expression level by 10- to 20-fold or more in multiple lots of cells following stimulation from either IL-4 or IL-13, which are allergy related cytokines and are genes that are closely related to allergies. There is a strong possibility that genes having such characteristics are the essential cause of allergic symptoms in a bronchial asthmatic attack. Therefore, the indicator genes provided by this invention become a useful indicator to know reliably whether the bronchial asthmatic attack was caused by allergic symptoms. By enabling reliable diagnosis of bronchial asthmatic attack caused by allergies, accurate treatment can be selected at an early stage.

[0086] IL-4 and IL-13 are important factors that enhance allergic reactions. Therefore, genes whose expression increases accompanying stimulation by these factors may accomplish important roles in pathologic formation of allergic symptoms. Furthermore, indicator proteins provided by this invention all showed clear elevation of their measured values in the blood of patients affected with bronchial asthmatic attack. Studies focusing on changes of gene expression level accompanying IL-4 or IL-13 stimulation are not novel. However, any genes provided by this invention are genes observed to show elevation of concentration in the blood of patients affected with bronchial asthmatic attack. Therefore, in contrast to known allergy related genes obtained by a similar approach, the indicator genes of this invention can be thought to be genes accomplishing important roles in bronchial asthmatic attack. These findings elucidated by the inventors provide evidence that the existence of indicator genes of this invention is indispensable to bronchial asthmatic attack.

[0087] The expression level of indicator genes provided by this invention can be easily known, regardless of the type of allergen. Therefore, the pathology of an allergic reaction can be understood overall.

[0088] Additionally, the method of testing for allergies of this invention has low invasiveness towards patients since analysis of expression level can be carried out using a biological sample as the sample. Furthermore, regarding gene expression analysis, highly sensitive measurements are possible using small amounts of samples. Year after year, the advances in gene analysis technology include high throughput methods and a decrease in cost. Therefore, in the near future the method of testing for allergies of this invention is expected to become an important bed-side diagnostic method. In this sense, there is a high diagnostic value of these pathology-related genes.

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45 <400> 13
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50 **Claims**

1. A method of testing for bronchial asthmatic attack using SCCA1 and/or SCCA2 as an indicator gene, said method comprising the steps of:

- 55 (a) measuring the expression level of the indicator gene in a biological sample from a subject, and
 (b) comparing the expression level measured in (a) with that in a biological sample from a living body not affected by bronchial asthma.

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2. The method of claim 1, wherein the biological sample is blood.
3. The method of claim 1, wherein the expression level of the gene is measured by PCR of the cDNA for the gene.
- 5 4. The method of claim 1, wherein the expression level of the gene is measured by detecting a protein encoded by the gene.
- 10 5. A reagent for testing for bronchial asthmatic attack, said reagent comprising an oligonucleotide that is at least 15 nucleotides long and that has a nucleotide sequence complementary to a polynucleotide having a nucleotide sequence of the SCCA1 and/or SCCA2 gene or to a complementary strand thereof.
- 15 6. A reagent for testing for bronchial asthmatic attack, said reagent comprising an antibody recognizing a peptide having an amino acid sequence of SCCA1 and/or SCCA2 protein.

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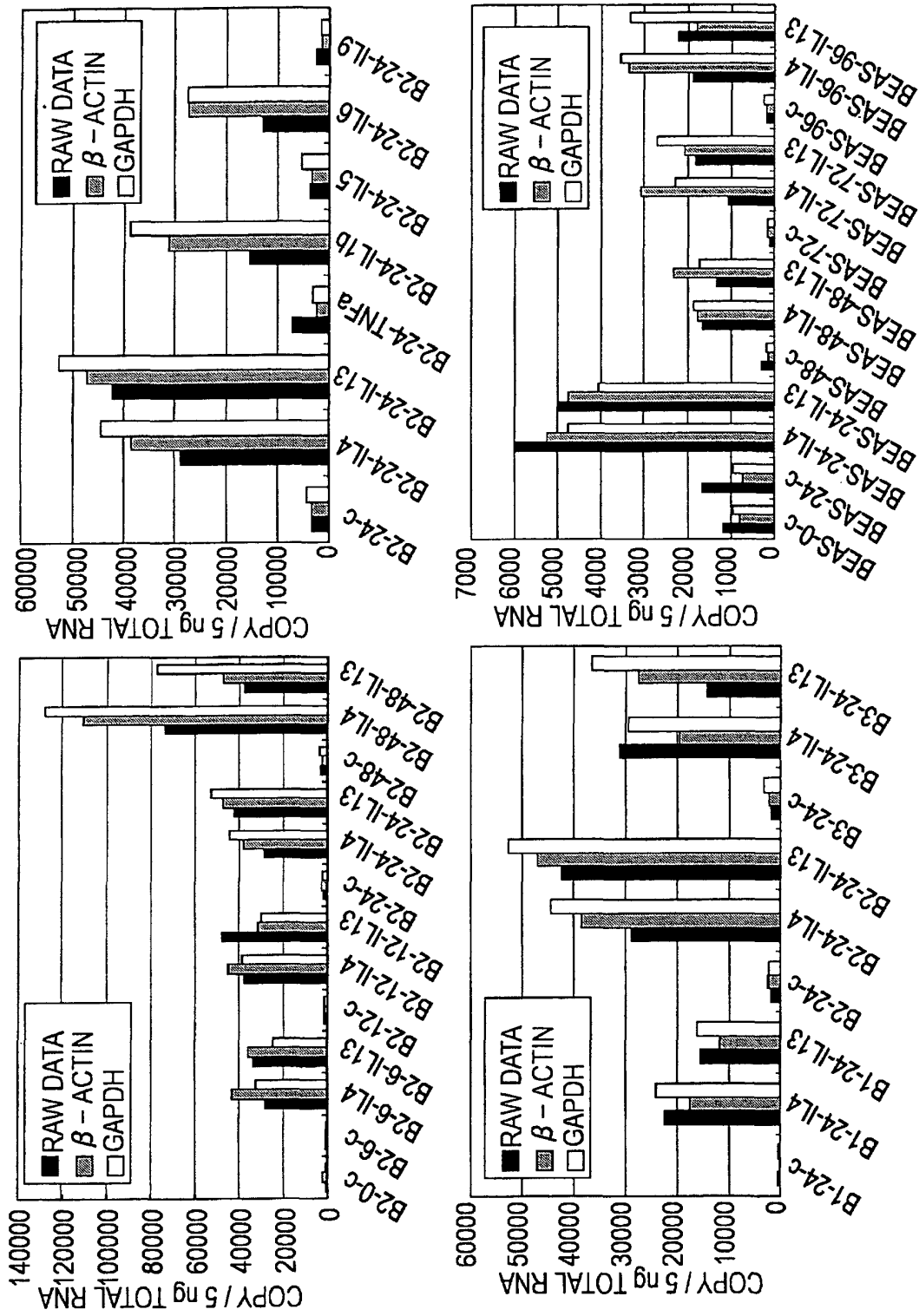


FIG. 1

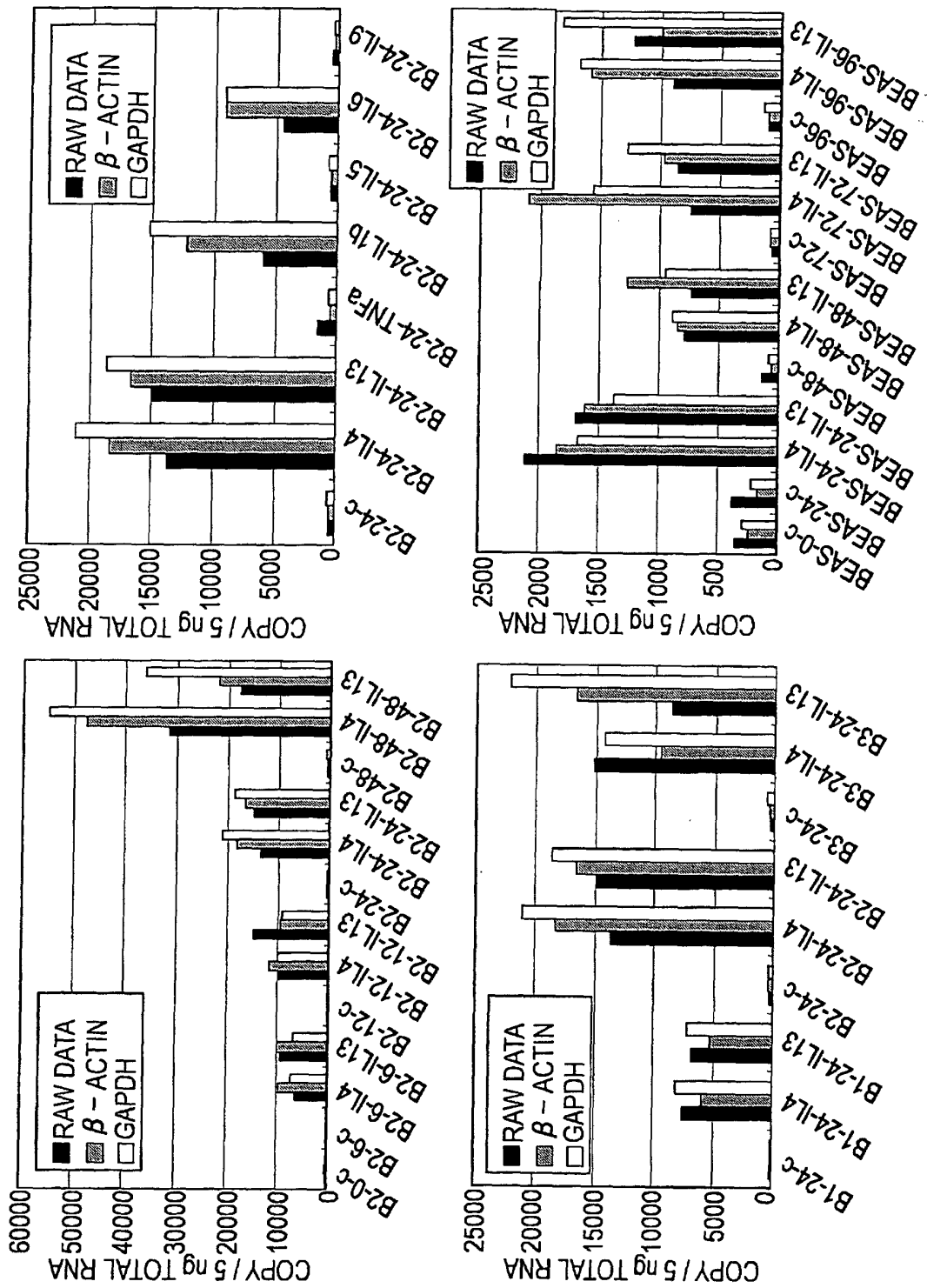


FIG. 2

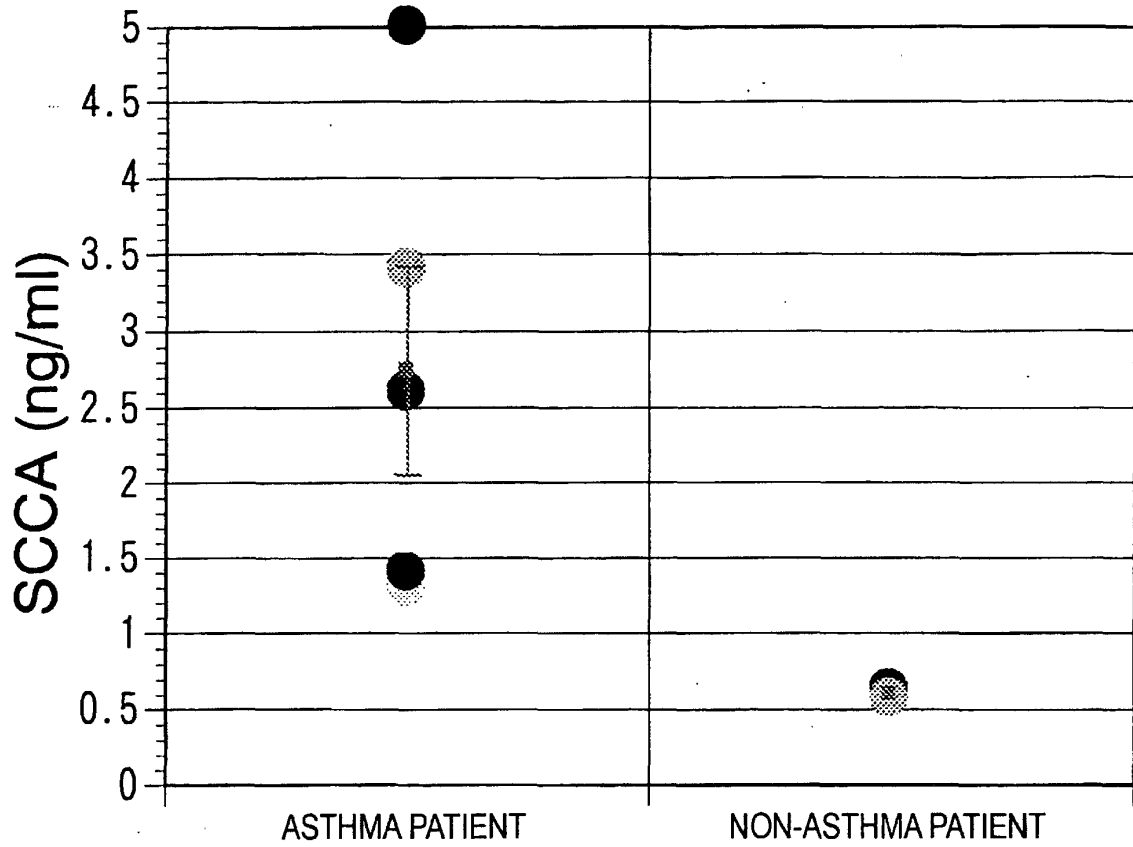


FIG. 3

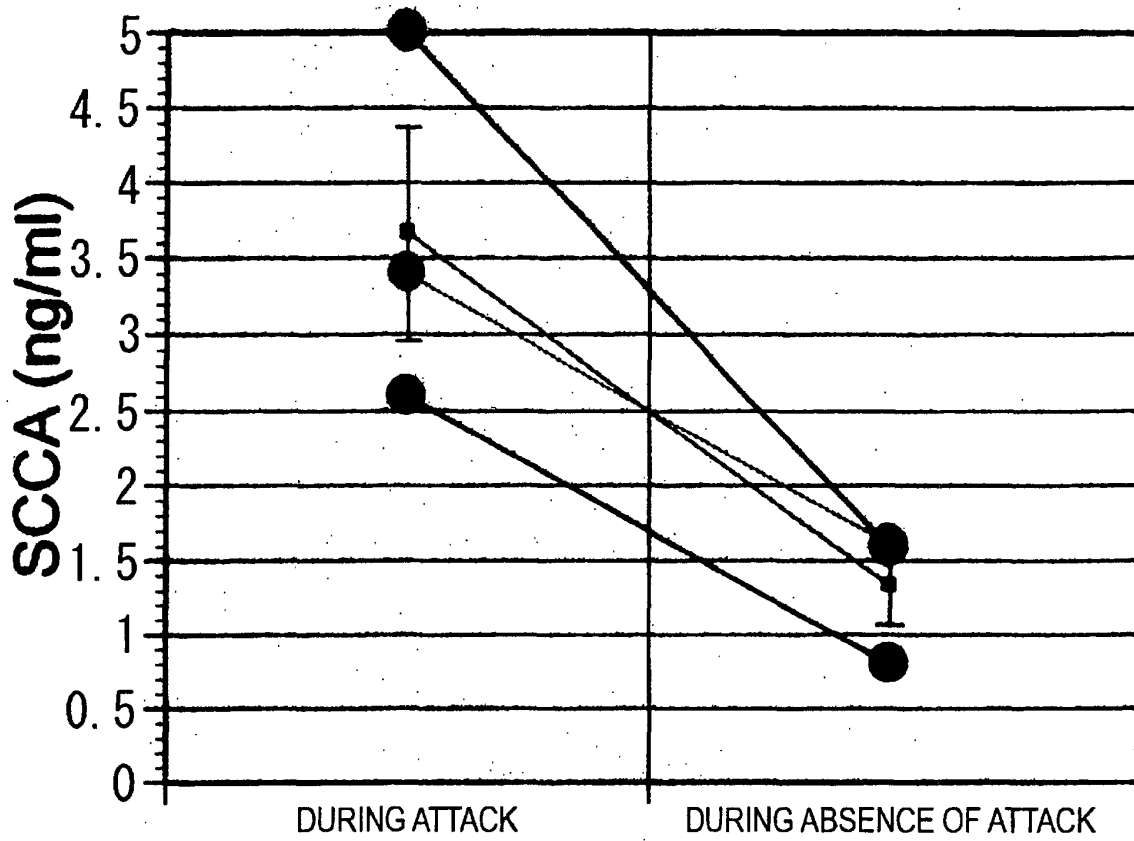


FIG. 4

INTERNATIONAL SEARCH REPORT		International application No. PCT/JP02/07918
A. CLASSIFICATION OF SUBJECT MATTER Int.Cl ⁷ C12Q1/68, C07K16/18, G01N33/53		
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) Int.Cl ⁷ C12Q1/68, C07K16/18, G01N33/53		
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 practicable, search terms used) CA (STN), MEDLINE (STN), BIOSIS (STN), WPIDS (STN), JICST (JOIS)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Kenji IZUHARA, "Microarray Ho o Mochiita Kikanshi Zensoku Kanren Idenshi Kensaku no Kokoromi", Arerugi Men'eki (2001 Sep.), Vol.8, No.10, pages 1148 to 1152	1-6
A	Derek J. Symula. et al., Functional screening of an asthma QTL in YAC transgenic mice., Natl.Genet. (1999), Vol.23, No.2, pages 241 to 244	1-6
A	Shick Charles, et al., Cross-class inhibition of elastolytic cycteine proteinases by the human serpin SCCA1., Pediatric Research (1997), Vol.41, No.4 (Part 2), page 306A	1-6
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 06 September, 2002 (06.09.02)	Date of mailing of the international search report 24 September, 2002 (24.09.02)	
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer	
Facsimile No.	Telephone No.	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP02/07918

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NOGUCHI E. et al., Haplotypes of the 5' region of the IL-4 gene and SNPs in the intergene sequence between the IL-4 and IL-13 genes are associated with atopic asthma., Hum. Immunol. (2001), Vol.62, pages 1251 to 1257	1-6

Form PCT/ISA/210 (continuation of second sheet) (July 1998)

专利名称(译)	支气管哮喘的检测方法		
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申请号	EP2002751829	申请日	2002-08-02
申请(专利权)人(译)	GENOX研究, INC.		
当前申请(专利权)人(译)	GENOX研究, INC.		
发明人	OHTANI, NORIKO, PHARMACEUTICAL RESEARCH LABORATORY MATSUI, KEIKO, TOKUMAJYUTAKU 433, 1594 YOSHIDA, NEI, C/O MEDICINAL GENOMIX INC. SUGITA, YUJI C/O YAMANOUCHI PHARMACEUTICAL CO.LTD. HAMASAKI, YUHEI, C/O DEPARTMENT OF PEDIATRICS IZUHARA, KENJI, DEPT. OF BIOMOLECULAR SCIENCES		
IPC分类号	C12Q1/68 G01N33/68 C07K16/18 G01N33/53		
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其他公开文献	EP1422297A1		
外部链接	Espacenet		

摘要(译)

通过用IL-4或IL-13刺激呼吸道上皮细胞,其表达在多个细胞中大大改变的SCCA1和SCCA2基因被证明是过敏相关基因。此外,阐明了这些基因在伴随支气管哮喘发作的血液中的表达产物的升高。本发明提供了一种测试支气管哮喘发作的方法,该方法使用这些基因在生物样品中的表达水平作为指标。