Title: PROTEIN P14 WITH ANTI-CANCER AND ANTI-ALLERGY ACTIVITY AND PHARMACEUTICAL COMPOSITION COMPRISING THE SAME

Abstract: Disclosed are protein p14 which exhibits anti-cancer and anti-allergy activity, and a pharmaceutical composition for the treatment, management, alleviation, improvement or prevention of cancer and/or allergic diseases, comprising the same. The composition guarantees excellent anticancer effects with minimal side effects. Protein p14 induces the synthesis of IFN-γ and regulates the production of IL-4, thus exhibiting preventive or therapeutic effects on allergic diseases.
Description

Title of Invention: PROTEIN P14 WITH ANTI-CANCER AND ANTI-ALLERGY ACTIVITY AND PHARMACEUTICAL COMPOSITION COMPRISING THE SAME

Technical Field

[1] The present invention relates to protein pl4 which exhibits anti-cancer and anti-allergy activity, and a pharmaceutical composition for the treatment, management, alleviation, improvement or prevention of cancer and/or allergic diseases, comprising the same.

[2] Background Art

[3] Cancer is a disease which is the main cause of human deaths around the world. Although the survival of cancer patients has increased with the development of new types of chemotherapy, neither the onset nor the severity of cancer has decreased.

[4] Cancer is an aggregate of cells characterized by unregulated cell growth and proliferation due to the loss of differentiation ability. Carcinogenesis is explained by a multistep process involving 5-8-steps of alterations of oncogenes and tumor suppressor genes. The activation of an oncogene induces the aberrant proliferation of cells whereas a tumor suppressor gene, when activated, initiates the operation of a cell death program in which specific cells are programmed to perish, thereby preventing cells from becoming cancerous.

[5] Because they are adapted to kill the cells that rapidly proliferate, most of the currently used anti-cancer agents cannot selectively remove cancer cells, but have an inhibitory influence on the normal cells that outgrow cancer cells, like the epithelial cells of digestive organs, or dermal papilla cells. Chemotherapy with such anti-cancer agents damages the normal cells as well, with the concomitant generation of side effects such as vomiting, hair loss, etc. In addition, conventional anti-cancer agents destroy lymphocytes, which rapidly proliferate during an immune response to pathogens. Accordingly, persons who are receiving chemotherapy are susceptible to bacterial or viral infections and have rather a decreased potential to control cancer cells. Further, the functioning of their bone marrow is weakened because conventional anti-cancer agents induce myelosuppression.

[6] There is therefore a pressing need for an anti-cancer agent that is effective at preventing the onset of cancer as well as treating cancer, with minimal toxicity.

[7] About 20% of the world population suffers from allergic disorders including asthma, allergic rhinitis, food allergy, atopic dermatitis and hypersensitivity. Anti-histamine
agents or steroid drugs, used as therapeutics for allergy, may incur significant side effects. Thus, research has been made to develop a therapeutic for allergy disorders which is therapeutically effective without side effects.

[8] **Disclosure of Invention**  

**Technical Problem**  

[9] It is therefore an object of the present invention to overcome the problems encountered in the prior art and to provide a novel protein having anticancer activity.

[10] It is another object of the present invention to provide a pharmaceutical composition for the prevention or treatment of cancer, which exhibits high therapeutic efficiency with side effects minimized.

[11] It is a further object of the present invention to provide a food composition useful as an aid in the treatment or prevention of cancer.

[12] It is still a further object of the present invention to provide a novel protein having an anti-allergy activity.

[13] It is still another object of the present invention to provide a food composition useful as an aid for the treatment, management, alleviation, improvement or prevention of allergic diseases.

[14] **Solution to Problem**  

[15] In accordance with an aspect thereof, the present invention provides protein pl4, represented by the amino acid sequence of SEQ ID NO: 1 or 3, having anti-cancer activity.

[16] In accordance with another aspect thereof, the present invention provides an anticancer pharmaceutical composition, comprising protein pl4 having the amino acid sequence of SEQ ID NO: 1 or 3.

[17] In accordance with a further aspect thereof, the present invention provides a pharmaceutical composition for the treatment or prevention of cancer, comprising a gene (nucleic acid) coding for the protein pl4.

[18] In accordance with still a further aspect thereof, the present invention provides a food composition useful as an aid for the treatment or prevention of cancer.

[19] In accordance with still another aspect thereof, the present invention provides protein pl4, represented by the amino acid sequence of SEQ ID NO: 1 or 3, having an anti-allergy activity.

[20] In accordance with yet a further aspect thereof, the present invention provides a gene coding for the protein pl4.

[21] In accordance with yet another aspect thereof, the present invention provides a re-
combinant vector carrying a gene coding for the protein pl4.

In accordance yet still a further aspect thereof, the present invention provides a transformant anchoring the recombinant vector therein.

In accordance with yet still another aspect thereof, the present invention provides a pharmaceutical composition for the treatment or prevention of allergic diseases, comprising the protein pl4.

**Advantageous Effects of Invention**

As described hitherto, the present invention addresses a pharmaceutical composition for the treatment or prevention of cancer, comprising a novel protein derived from lactic acid bacteria, as an active ingredient. In addition to exhibiting excellent preventive or therapeutic effects on cancer, the composition of the present invention creates neither of cytotoxicity nor causes side effects because a protein derived from probiotic organisms or dead bacteria beneficial to humans is used as an active ingredient. Further, the active ingredient can maintain its pharmaceutical effects for a long period of time. Thus, the composition may be safely applied to a food composition in addition to a pharmaceutical composition.

Also, the present invention addresses a composition for the prevention or treatment of allergic diseases, comprising a novel protein derived from *Lactobacillus* as an active ingredient. Protein pl4, used in the present invention, induces the synthesis of IFN-γ and regulates the production of IL-4, thus exhibiting preventive or therapeutic effects on allergic diseases. Derived from probiotic organisms or dead bacteria beneficial to humans, the active ingredient is free of cytotoxicity and does not cause side effects. In addition, its pharmaceutical efficiency lasts for a long period of time. Thus, it is safely applicable to a food composition in addition to a pharmaceutical composition.

Moreover, the present invention provides a gene coding for the protein pl4 which has anti-allergy and/or anti-cancer activity, with which recombinant protein pl4 can be produced in a large quantity.

**Brief Description of Drawings**

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 is an FPLC chromatogram showing 10 ~ 100 kDa fractions of a culture of *Lactobacillus paracasei*;

FIG. 2 is a graph showing anti-cancer activity of fractions 16-19 of a culture of *Lactobacillus paracasei*;
FIG. 3 shows the identification of proteins having anticancer activity as assayed by silver staining;

FIG. 4 shows Blast search results for the identification of anticancer proteins through amino acid sequence alignment;

FIG. 5a shows schematic structural diagrams of primers designed on the basis of deduced CDS areas;

FIG. 5b shows PCR products of genes coding for 13 kDa, 14 kDa, and 49 kDa proteins which will be inserted into E. coli expression vectors;

FIG. 6 shows the cloning of genes coding for 13 kDa, 14 kDa, and 49 kDa proteins into vectors, using restriction enzymes and T4 ligase;

FIG. 7 shows SDS-PAGE results of recombinant proteins overexpressed in E. coli colonies by IPTG induction;

FIG. 8 shows the expression of 13 kDa, 14 kDa, and 49 kDa proteins over time in E. coli colonies under IPTG induction;

FIGS. 9a and 9b show isolation, purification and dialysis results of protein pl4 from E. coli cultured on a large scale;

FIG. 10 shows similarity between the recombinant proteins P13 and p14 in terms of amino acid sequence;

FIGS. 11a and 11b show anti-cancer activity of P13 and p14 against colorectal cancer cell lines HT-29 and DLD-1, as measured by MTT assay;

FIG. 12 shows the cytotoxicity of protein pl4 in terms of the cell survivability of mouse macrophages;

FIGS. 13a and 13b shows immunopotentiation of protein pl4 in mouse splenocytes;

FIG. 14 shows feed intake of F344/N mice treated with or without p14 after the induction of large intestine cancer by the carcinogen DMH;

FIGS. 15a and 15b show counts of AC (aberrant crypt) and ACF (aberrant crypt foci) formed in the colonic mucosa of mice treated with or without protein pl4 after cancer induction by DMH;

FIG. 16 shows the growth of tumors in nude mice xenografted with the human large intestine cancer cell line DLD-1, which is inhibited 10% more by pl4 than by the control;

FIG. 17 shows sizes of tumors in nude mice xenografted with the human large cancer intestine DLD-1 before administration with pl4 and after four rounds of administration with pl4;

FIG. 18a shows INF-γ levels in activated mouse splenocytes treated with the protein pl4 of the present invention, and FIG. 18b shows IL-4 levels in activated mouse splenocytes treated with the protein pl4 of the present invention;

FIGS. 19a to 19c show anti-allergy activity of the protein pl4 of the present
invention in terms of NO production in mouse macrophages over time;

FIG. 20 shows in vivo anti-allergy effects of pl4 on atopy-induced mice; FIG. 21 shows serum IgE levels in atopy-induced mice after treatment with the protein pl4 of the present invention; and

FIG. 22 shows serum 11-4 levels in atopy-induced mice after treatment with the protein pl4 of the present invention.

Best Mode for Carrying out the Invention

As used herein, the term "cancer" is intended to include tumors, neoplasms, and malignant tissues or cells. Examples of the cancer include cancer of the large intestine, lung cancer, small cell lung cancer, stomach cancer, liver cancer, blood cancer, bone cancer, pancreatic cancer, skin cancer, head and neck cancer, intradermal or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, perianal cancer, colon cancer, breast cancer, fallopian tube carcinoma, endometrial cancer, uterine cervical cancer, vaginal cancer, vulvar cancer, Hodgkin's disease, esophageal cancer, small intestine cancer, endocrine gland cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue sarcoma, urethral cancer, penis cancer, prostate cancer, chronic or acute leukemia, lymphoma, bladder cancer, renal cancer, ureteral cancer, renal cell carcinoma, renal pelvis cancer, CNS tumor, primary CNS lymphoma, spinal cord tumor, brainstem glioma, pituitary adenoma and a combination thereof.

The term "allergy," as used herein, means INF-y-mediated allergy. In this regard, rhinitis, sinusitis, atopy, asthma, hypersensitive pneumonia, extrinsic allergic alveolitis, conjunctivitis, hives, pruritus, sun allergy, eczema, dermatitis, anaphylaxis, angioedema, allergic headache and migraine, and certain digestive troubles fall within the scope of the term allergy.

According to one aspect thereof, the present invention addresses anti-cancer protein pl4 having the amino acid sequence of SEQ ID NO: 1 or 3. According to another embodiment, the present invention addresses a pharmaceutical composition for the treatment of cancer, comprising the protein pl4 as an active ingredient.

Protein pl4 has a molecular weight of about 14 kDa. The composition of the present invention may comprise a functional equivalent of protein pl4. As used herein, the term "functional equivalent" is intended to refer to a protein which shares an amino acid sequence homology of 70% or greater, preferably 80% or greater and more preferably 90% or greater with SEQ ID NO: 1 or 3 as a result of amino acid residue addition, substitution or deletion, exhibiting a physiological function substantially identical to that of the protein of SEQ ID NO: 1 or 3. The term "substantially identical physiological function," as used herein, means anti-cancer and/or anti-allergy activity
in animals.

In one embodiment of the present invention, protein pl4 is derived from *Lactobacillus casei, Lactobacillus paracasei* or *Lactobacillus rhamnosus*. Illustrative, non-limiting examples include Lactobacillus paracasei LPC4 (KCTC 11866BP). The protein pl4 derived from Lactobacillus rhamnosus has the amino acid sequence of SEQ ID NO: 3, which shares a homology of about 90% with that of SEQ ID NO: 1 and which can be encoded by the nucleotide sequence of SEQ ID NO: 4.

The protein pl4 derived from *Lactobacillus casei, Lactobacillus paracasei* or *Lactobacillus rhamnosus* is recognized by the epidermal growth factor receptor (EGFR), present on the cell surface, to enhance the Akt-mediated signaling pathway or the JNK- or MAPK-mediated signaling pathway.

Functioning to increase the release of interferon gamma and suppress the production of interleukin-4, protein pl4 can be used for preventing or treating allergic diseases.

In accordance with another aspect thereof, the present invention addresses an anti-cancer pharmaceutical composition comprising a gene (nucleic acid) coding for the protein pl4 as an active ingredient. The gene includes genomic DNA and cDNA so long as it encodes protein pl4. Preferably, the gene comprises the nucleotide sequence of SEQ ID NO: 2 or 4.

Also, a mutant of the nucleotide sequence may fall within the scope of the present invention. For example, the gene may have a nucleotide sequence which shares a homology of 70% or higher, preferably 80% or higher and more preferably 90% or higher with the nucleotide sequence of SEQ ID NO: 2. The term "percentage of sequence homology" as used herein in connection with a polynucleotide refers to a value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) in order to optimally align the two sequences.

In accordance with a further aspect thereof, the present invention addresses a recombinant expression vector carrying a protein pl4-encoding gene (nucleic acid). The recombinant expression vector may be constructed by linking a gene of interest to a recombinant vector used in the art in a conventional manner. This expression vector may be a plasmid or a viral vector. Plasmid expression vectors are approved for use in gene delivery to humans by the FDA. Plasmid expression vectors can deliver DNA directly to human cells (Nabel, E. G., et al., Science, 249:1285-1288, 1990). Unlike viral vectors, plasmid DNA can be advantageously purified with homogeneity. Mammalian expression plasmids known in the art may be used as expression vectors in the present invention. Examples of the expression vector useful in the present invention include,

[63] The viral expression vector carrying the nucleic acid, useful in the present invention, may include those derived from a retrovirus, adenovirus, herpes virus and avipox virus, but is not limited thereto.

[64] In accordance with still a further aspect thereof, the present invention addresses a transformant prepared by introducing the recombinant vector into a host cell. The transformation may be achieved using a conventional method. For example, a recombinant vector can be introduced into target cells using transient transfection, microinjection, transduction, cell fusion, calcium phosphate precipitation, liposome-mediated transfection, DEAE dextran-mediated transfection, polybrene-mediated transfection, electroporation, a gene gun or a well-known method (Wu et al., J. Bio. Chem., 267:963-967, 1992; Wu and Wu, J. Bio. Chem., 263:14621-14624, 1988).

[65] No particular limitations are imposed on the host cells to be formed into transformants so long as they can be suitably transformed by the recombinant vectors of the present invention. Various cells known in the art, whether natural or synthetic, may be employed. For example, E. coli BL21 and BL21 (DE3) may be used, with a preference for E. coli BL21 (DE3). The transformed E. coli can be cultured using a method that is commonly used to culture E. coli in the art.

[66] According to still another aspect thereof, the present invention addresses an anti-cancer pharmaceutical composition, comprising protein pl4 having the amino acid sequence of SEQ ID NO: 1 or 3 as an active ingredient. The anti-cancer pharmaceutical composition may comprise a pharmaceutically acceptable excipient, diluent, carrier or buffer in addition to the active ingredient protein pl4, which exhibits anti-cancer activity.

[67] According to yet a further aspect thereof, the present invention addresses an anti-allergy pharmaceutical composition comprising (a) protein pl4, having an anti-allergy activity; and, (b) a pharmaceutically acceptable excipient, diluent, carrier or buffer.

[68] For pharmaceutically acceptable additives suitable for use in the present invention, including buffer, diluents, vehicles, supplements, and other excipients, Remington's Pharmaceutical Sciences (19th ed., 1995) may be referred to. Suitable agents may be employed in the composition to achieve various other purposes. For example, a buffer, preservatives, co-solvents, oils, wetting agents, softeners, stabilizers or antioxidants may be used. Examples of aqueous preservatives useful in the present invention include sodium bisulfate, sodium thiosulfate, benzalconium chloride, chlorobutanol, thimerosal, ethyl alcohol, methyl paraben, polyvinylalcohol, benzyl alcohol and phenylethyl alcohol. These agents may be present in an amount of about 0.001 to about 5 wt% individually. Aqueous buffers suitable for use in the present invention are
sodium carbonate, sodium borate, sodium phosphate, sodium acetate and sodium bicarbonate, the desired routes of administration of which have been approved by the FDA. These agents may be present in an amount sufficient to keep the pH of the system within the range of about 2 to about 11. Thus, the buffer may be used in an amount of about 5 wt % based on the total weight of the composition. In addition, electrolytes such as sodium chloride and potassium chloride may be contained in formulations.

[69] Medical products to which the protein pl4 of the present invention is applied to may further comprise one or more active ingredients exhibiting anticancer activity identical or similar to that of the protein pl4.

[70] For clinical use, the protein pl4 may be orally administered and may be formulated into general medical preparations. In practical clinics, the protein pl4 of the present invention may be used in various oral dosage forms. Solid forms for oral administration include tablets, pills, powders, granules, capsules, etc.

[71] The method for preparing the anticancer and/or anti-allergy pharmaceutical composition comprises formulating protein pl4 with a pharmaceutically acceptable excipient, diluents, carrier or buffer. In one embodiment, the preparation method of the present invention comprises formulating protein pl4, at least one anticancer agent and a pharmaceutically acceptable excipient, diluent, carrier, or buffer. Examples of the anticancer agent include ciplatin, doxorubicin and etoposide, but are not limited thereto.

[72] According to a method well known to those skilled in the art, the pharmaceutical composition of the present invention may be formulated, together with pharmaceutically acceptable carriers and/or excipients, into unit dose forms or may be included within a multiple dose package.

[73] The anticancer pharmaceutical composition or the anti-allergy pharmaceutical composition of the present invention comprises a pharmaceutically effective amount of protein pl4. The term "pharmaceutically effective amount," as used herein, refers to an amount sufficient to treat or prevent cancer. The pharmaceutically effective amount of protein pl4 in accordance with the present invention is within the range of 0.1 ~ 100 mg/day/ kg of body weight, and preferably 12 mg/day/kg of body weight. The dose of protein pl4 may vary depending on various factors including the route of administration, the severity of illness, and the gender, weight and age of patient.

[74] In accordance with another embodiment, the composition comprising protein pl4 of the present invention may be applied to foods including beverages helpful in the treatment of cancer and/or allergic diseases. Foods to which the protein pl4 of the present invention is applicable are beverages, teas, vitamin complex products, health aid foods, etc. The protein pl4 itself, isolated from lactic acid bacteria, creates almost no toxicity and side effects, so that it can be safely used for a long period of time for
prophylactic purposes.

[75]

**Mode for the Invention**

[76] A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

[77]

**EXAMPLES**

[78]

**EXAMPLE 1: Isolation and Identification of Protein pl4 and Production of Recombinant Protein**

[79]

[80] 1-1. Isolation and Purification of Protein with Anticancer Activity from a Culture of *Lactobacillus paracasei*

[81]

[82] Proteins were separated into elution fractions on a phenyl sepharose column loaded with a culture of the Lactobacillus paracasei LPC4 strain (KCTC 11866BP). For this, first, the Lactobacillus paracasei strain was cultured and concentrated. Proteins with anticancer activity were purified from the cell concentrate by FPLC (fast protein liquid chromatography, Sephadex 75) on a Sephadex 75 column (fractionation range Mr = 3,000 ~ 70,000) eluting with a buffer (0.05 M phosphate and 0.15 M NaCl, pH 7.2) at a flow rate of 0.5mL/min, with 1 mL of the sample (2-4 mg/mL) loaded thereinto. Proteins with anticancer activity were found in 3-100 kDa and 10-30 kDa fractions after ultrafiltration (FIG. 1).

[83]

**1-2. Determination of Anticancer Activity Fractions and Identification of Amino Acid Sequences thereof**

[84]

[85] Fraction Nos. 16-19 were assayed for anticancer activity. In this regard, 900 µL aliquots of each of the cancer cell lines including stomach cancer (AGS), lung cancer (A549), breast cancer (MCF-7), ovarian cancer (SKOV-3) and colorectal cancer (LoVo) were each put into a well of a 24-well plate and incubated for 24 hours (37°C, 5% C02). To each well was added 100 µL of each of the purified fractions before incubation for an additional 24 hours (37°C, 5% C02). Subsequently, each well was supplemented with 100 µL of an MTT solution (0.5mg/mL) and incubated for 4 hours (37°C, 5% C02). After removal of the supernatant, 300 µL of DMSO (dimethyl sulfoxide) was added to each well to dissolve the produced formazan, followed by measuring absorbance at 540 nm in an ELISA reader. From the absorbance values, inhibition activity of the protein fractions against cancer cell growth were calculated
according to the following equation and expressed as inhibition ratio (%). The results are shown in FIG. 2. Each fraction was recovered in an amount of 3 mL/fraction from FPLC (Sephadex 75) (0.5ml/min).

\[ \text{Inhibition ratio(\%) = } (1 - \frac{T}{QX100}) \]

(T: absorbance in the presence of sample, C: absorbance in the absence of sample)

Proteins in the 3-100 kDa fractions were examined for anticancer activity. As can be seen in FIG. 2, the control did not exhibit cytotoxicity against the cancer cells whereas proteins in fraction Nos. 16 to 19 of the 10-30 kDa fractions were found to have anti-cancer activity.

1-3. Identification of Anticancer Protein p14

Proteins were separated at pI4 and p44 from fraction Nos. 16-19, as assayed by silver staining and Coomassie blue 250 staining (FIG. 3). They were characterized and their amino acid sequences were determined. In this context, liquid chromatography/mass spectroscopy of the proteins detected by staining yielded three peaks at p49, p14 and pI3. The 49-kDa protein is a cell wall-associated hydrolase and showed a similarity of 95% or higher with a cell wall-associated hydrolase. The protein pI4 is a hypothetical protein whose function remained unknown, with a similarity of 95% or higher with a hypothetical protein LSEI_0419 (FIG. 4).

1-4. Production of Recombinant Proteins

A. Design and Construction of Restriction Site-Containing Primers

To clone genes coding for the 13 kDa, the 14 kDa and the 49 kDa protein into the E. coli expression vector pRSET A (Invitrogen), primers containing BamHI and XhoI sites were designed and synthesized as shown in FIG. 5a. In preparation for counteracting the likelihood of not recognizing the restriction sites due to mutation, one or two bases were added in close proximity to the restriction sites. The reading frame was also considered when designing the primers. The genes coding for 13 kDa, 14 kDa and 49 kDa proteins were amplified using the primers and cloned into a pRSET A vector (FIG. 5a).

B. PCR for Amplification of CDS Area to Be Inserted into E. coli Expression Vector

Using the primers, the PCR products of the CDS areas were amplified with annealing at 55°C. For amplification, a reaction mix containing 3 µL of a substrate, (10 ng/µL), primers (10 pM), MgC12 (25mM), dNTP(2.5mM), Taq polymerase (5 U/µL) and 10× buffer was employed. PCR started with initial denaturation at 95°C for 2 min and was performed with 30 thermal cycles of denaturing at 95°C for 20 sec, annealing at 55°C for 35 sec and extension at 72°C for 20 sec. High fidelity polymerase Taq (Invitrogen) was employed for the PCR, with the DNA of Lactobacillus casei serving as a template.
The PCR products were measured to be 369, 372, and 1494 bp long, which were the same as the sizes of the three proteins, respectively (FIG. 5b).

C. Transformation

pRSET and the PCR products were digested with the restriction enzymes BamHI, XhoI and EcoRI at 37°C for 2 hours, and separated on agarose gel by electrophoresis. The vector was ligated with the inserts. For this, the vector was used at an intensity ratio of 1:3 with the insert as measured by EtBr staining. The ligated vector was transformed into competent cells (DH5a) and incubated to form a total of 22 colonies (FIG. 6). Of them, nine (colony Nos. 6, 7, 8, 10, 11, 13, 18, 20 and 21) were found to have exact inserts. Base sequencing analysis was performed on 13 kDa, 14 kDa, and 49 kDa recombinant protein-expressing plasmids thus obtained, using a T7 promoter primer to confirm the transformation.

D. Expression of 13 kDa, 14 kDa, and 49 kDa Recombinant Proteins

After the BL21 strain was transformed with the recombinant plasmid carrying a gene coding for the 13 kDa, the 14 kDa or the 49 kDa protein, a selection was made of E. coli colonies that showed a high expression level of the protein. To this end, 7 or 8 colonies were induced for 4 hours in the presence of 1 mM IPTG to express the proteins, followed by electrophoresis on 15% bisacrylamide gel for 90 min at 80 V. (Loading volume: 1 mL induction, 25 μL IX sample buffer Boiling 5 min, 10 μL loading). As a result, high levels of the proteins were detected in colony Nos. 13-1, 14-4 and 49-8 (FIG. 7).

E. Induction of Selected Colonies Under Various Conditions

The 14 kDa recombinant protein was observed to be stably expressed after having been induced for 1 hour with IPTG, irrespective of the concentration of IPTG. The colonies were O/N cultured in LB broth. Of 3 mL of the O/N culture, 0.5 mL was inoculated into 10 mL of fresh LB broth. The cells were grown for two or more hours to an OD600 of 0.5 before induction with IPTG. Samples were taken by time, and then run on 15% bisacrylamide gel for 90 min at 80 V by electrophoresis (Loading volume: 1 mL induction, 25 μL IX sample buffer, Boiling 5 min, 10 μL loading). Induction of the proteins of 13 kDa, 14 kDa, and 49 kDa over time showed that expression of only the 14 kDa recombinant protein was stable (FIG. 8).

F. Isolation and Purification of pL4 Recombinant Protein from Large-Scale Culture

Based on the result that only expression of the protein pL4 was stable (10 mL culture, 1 mM IPTG), the culture was scaled up (300 mL) and stable induction was made
therein (0.1 mM IPTG) (FIG. 9a). In addition, cells were harvested from the 300 mL culture and proteins were recovered from the cells using the BugBuster Master Mix (Merk). Purification using the His Taq BugBuster Ni-NTA His*Bind Purification Kit (Merk) gave 2 mL of a purified protein. SDS-PAGE of 20 µL of the purified protein demonstrated purification. In this regard, purification was confirmed by running a flow through the His Taq column, running a wash fraction, and separating an elution fraction on the gel and visualizing with Coomassie blue 250. The protein pl4 recovered many times in the same manner was observed to have a high purity after dialysis (FIG. 9b).

[112] G. Analysis of Amino Acid Sequence of pl4 Recombinant Protein (FIG. 10)

After electrophoresis on Coomassie gel, the band of the pl4 recombinant protein expressed in E. coli was recovered and stirred for 5 min in 100 µL of a destaining solution (50% MeOH/D.W.). Then, the destaining solution was completely removed. The residue was incubated for 20 min in 100 µL of 200 mM ABC (ammonium bicarbonate, pH 7.8) and then, the ABC was removed. The residue was vortexed for 2 min in 100 µL of acetonitrile and then, the acetonitrile was discarded. Again, the residue was vortexed for 2 min in 100 µL of ABC and then the ABC was discarded. Vortexing was also continued for 2 min with 100 µL of acetonitrile. This procedure was repeated three times in total. After acetonitrile was completely removed in a final step, the gel was dried at room temperature. To the dried gel was added 20 µL of trypsin (0.2 µg) which was incubated for 45 min in ice. The trypsin which remained unabsorbed into the gel was removed. The gel was incubated at 37°C for 12 hours in 70 µL of 50 mM ABC. GELoader Tip microcolumns packed with Poros R2 resins were prepared. A. 10 mL syringe was fitted to the tip so as to apply air pressure to the column. The microcolumns were equilibrated with 20 µL of 5% formic acid solution which was allowed to pass through the microcolumn by applying them using the syringe (equilibration). To the equilibrated microcolumns was loaded 30 µL of a solution of the peptide obtained by in-gel digestion (loading). Using the syringe, 20 µL of 5% formic acid solution was passed through the microcolumns (washing). Thereafter, the peptide was eluted with 1.5 µL of 50% methanol/49% H2O/1% formic acid solution by applying air pressure from the syringe (elution). With the aid of nano-electrospray needle (EconoTipTM, New Objective, USA), 1.5 µL of the eluate was applied to a Q-TOF source. The amino acid sequence of the separated proteins was examined using an MS instrument: Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (AB Sciex Instruments, CA 94404 USA). Three proteins 10, 14, and 14 were found to have the same amino acid sequence as that of pl4 (conducted by Protein Works).
Each of the colorectal cancer cell lines DLD-1 and HT-29 were plated into 96-well plates and incubated for 48 hours with various concentrations of the protein pl4 (10 ng/ml to 1000 ng/ml). For a negative control, the cells were treated only with 0.1% PBS alone while protein P13 isolated from lactic acid bacteria was used as a positive control. Then, the cells were disrupted in a lysis buffer. The cell lysate was incubated at 37°C for 50 min with MTT (3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide, Sigma, U. S. A.). Absorbance at 610 nm was measured in a microplate reader (Benchmark, BIO-RAD, Japan) to calculate cell survivability. The results are shown graphically in FIGS. 11a and 11b, which are for HT-29 and DLD-1, respectively. As can be seen in FIGS. 11a and 11b, protein pl3 did not exhibit inhibitory activity against the cancer cells whereas protein pl4 was excellent at inhibiting the growth of both DLD-1 and HT-29 cell lines in a dose-dependent manner at an inhibition rate of from about 20% to about 40%.

To examine whether the protein pl4 of the present invention is toxic to cells, an MTT assay was performed. After being overexpressed, the protein pl4 was assayed for cytotoxicity against the mouse macrophage cell line Raw264.7 while using P13 as a positive control. The cells were seeded at a density of 5x10^3 cells/well into 96-well plates, and P13 and pl4, purified after overexpression, were added to each well (at a concentration of from 10 ng/ml to 1000 ng/ml). After incubation for 48 hours, the cells were lysed in a lysis buffer and incubated at 37°C for 50 min with MTT (3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide, Sigma, U. S. A.). Absorbance at 610 nm was measured using a microplate reader (Benchmark, BIO-RAD, Japan) to calculate cell survivability. The results are shown in FIG. 12. As can be seen in FIG. 12, there is no difference in cell survivability between the test group and the control. Thus, protein pl4 was found to have no cytotoxicity, as measured by MTT assay (FIG. 12).

An examination was made of whether the protein pl4 of the present invention has anti-cancer immunoactivity. Balb/c splenocytes were seeded at a density of 2x10^6 cells/mL into 96-well plates and treated for 48 hours with various concentrations of the
target proteins P13 and pl4 (0.01 µg/mL to 10 µg/mL). After centrifugation, the supernatant thus obtained was analyzed using ELISA. A negative control was treated with 0.1% PBS devoid of protein pl4 while a positive control was treated with P13. Concentrations (pg/mL) of the cytokines INF-γ and IL-12 were measured and are shown in FIGS. 13a and 13b, respectively. As is understood from the data of FIGS. 13a and 13b, protein pl4 was observed to induce the expression of IL-12 and INF-γ at higher rates than did the positive control P13, indicating that protein pl4 has efficient anticancer immunoactivity.

EXPERIMENTAL EXAMPLE 4: In vivo Anticancer Activity Assay (MTT assay)

4-1. Induction of Neoplastic Polyps by 1,2-Dimethylhydrazine (DMH)

A solution of the carcinogen 1,2-dimethylhydrazine (DMH; Sigma, USA) in citrate buffer was subcutaneously injected at a dose of 30 mg/kg four times (twice a week for two weeks) into the head and neck area of F344/N rats to form neoplastic polyps which developed into colorectal cancer. The test material protein pl4 was mixed with powdered basic feedstuff and pelletized.

Rats were divided into four groups: one was administered with physiological saline (sham) and the other three groups were injected with carcinogen DMH in combination with administration with basic feedstuff (PC), pl3 (pl3) and pl4 (pl4), respectively. The rats were fed ad libitum with purified water and basic foodstuff. The test materials were administered at a dose of 200 µg/kg for 8 weeks, during which body weight and feed intake were recorded once a week. Clinical symptoms were monitored with the naked eye (FIG. 14).

After the injection of DMH, as can be seen in FIG. 14, feed intake was significantly reduced in all the test groups compared to the control (sham). The reduction of feed intake by DMH was considered attributable to the DMH-induced development of polyps which led to decreased digestibility. Relatively high feed intake rates were detected in the pl4-administered group. Administration pl4 alone allowed higher feed intake rates than administration with pl3 alone, indicating that pl4 is useful for the therapy of colorectal cancer.

4-2. Suppressive Activity of pl4 against DMH-induced Polyps

To examine the suppressive effect of pl4 on DMH-induced polyps, a postmortem examination was made on all subjects after administration of the test materials for 8 weeks. Polyps were examined on a predetermined size of the large intestine organ extending to the rectum above the anus. The excised large intestine tissue was maximally swelled in a mixture of 1:1 0.85% saline : 10% neutral formalin and then
pre-fixed for 30 min in 10% neutral formalin. It was thinly spread and attached to a filter membrane so as not to distort the shape, followed by post-fixation in 10% neutral formalin. The fixed large intestine was stained with 0.5% methylene blue to visualize aberrant crypts (AC) and aberrant crypt foci (ACF) which were counted under a 100x optical microscope. The counts are shown in FIGS. 15a and 15b, respectively. As can be seen in FIGS. 15a and 15b, ACF and ACF counts were significantly decreased in the pl4 group compared to the other test groups.

EXAMPLE 5: Anticancer Activity of pl4 in Xenograft Animal Model

The anticancer activity of pl4 was assayed in xenografted animals which had been prepared by subcutaneously injecting 1x10^7 cells of the human colon cancer cell line DLD-1 into nude mice. To this end, first, 1x10^7 cells/nude mouse/100 μL was implanted into a subcutaneous region. Ten days after implantation, the state of the tumor cells was examined and the animals in which the implanted tumor cells had stable growth were monitored. Before the occurrence of central necrosis, the animals in which the tumors rapidly grew with a sufficient supply of blood were sacrificed. Peripheral tumor regions where cell division was taking place vigorously were excised to obtain tumor fragments in a predetermined size (3x3x3 mm). Subsequently, the tumor fragment was implanted by inserting a trocar carrying the tumor fragment into the mice through an incision made to a length of about 4 mm on the lateral flank of the left hind leg. In this context, the end of the trocar was allowed to reach the rear lateral flank of the left frontal leg. The trocar was withdrawn while being lightly and rapidly rotated, whereby the tumor fragment was set in the target position. The incision was aseptically treated. The position of the tumor fragment was confirmed by touching the surface of the skin. Two or more observations were made of the growth of the tumor fragment and animals in which the tumor fragment was successfully engrafted were selected for further experiments.

pl4 was intravenously injected at a dose of 1 mg/kg four times in total (once per two days) into the xenografted animal models. The size of the tumor was measured every two days using a vernier caliper. From the measurements, the volume of tumor was calculated according to the following equation, and is shown in FIG. 16.

\[
\text{Volume of Tumor} = \frac{(\text{Length of Long Axis} \times (\text{Length of Short Axis})^2)}{2}
\]

After four rounds of administration, the tumors were excised and photographed as shown in FIG. 17.

As can be seen in FIGS. 16 and 17, pl4 was found to regulate, although not
thoroughly, the growth of the tumor so that the size of tumor in the pl4-administered group was remarkably reduced, compared to the control. The volume of the tumor was increased at a rate of 63.3% in the control and 49.6% (92.8→196.8mm2) in the pl4-administered group, indicating that the percent inhibition of pl4 was more than 10%.

[146] EXPERIMENTAL EXAMPLE 6: Anti-Allergic Activity of pl4
[147] A. Assay for Anti-Allergic Activity in Splenocytes
[148] Cells were separated from the spleen of Balb/c mice, and the splenocytes were seeded at a density of 2x10^6 cells/mL/well into well plates. Treatment with 1 μg/mL Concanavalin A (ConA) allowed the splenocytes to be activated as in allergic diseases. The cells were incubated for 48 hours with various concentrations of the target proteins pl3 and pl4. After centrifugation, the supernatant thus formed was subjected to ELISA assay. For control comparison, the splenocytes were treated with PBS devoid of pl4. In the case of the quantitative analysis of IL-4, the cells were incubated in the presence of a mixture of l:1 pl3 : pl4 (MIX), as well. INF-γ and IL-4, which are respectively representative of the cytokines of T helper (Th1) and Th2 cells, were quantitatively analyzed. The results are shown in FIGS. 18a and 18b, respectively. As seen in FIGS. 18a and 18b, the level of IL-4 was slightly reduced in the pl4-administered group compared to the group treated with Con A alone while the level of INF-γ was increased by pl4. This data indicates that the protein pl4 of the present invention exhibits anti-allergic activity by increasing the level of INF-γ and inhibiting the production of IL-4.

[151] B. Assay of pl4 for Anti-Allergic Activity Using Mouse Macrophage Cells
[152] To confirm the anti-allergic activity of the lactic acid bacteria-derived pl4 of the present invention, the production of NO by mouse macrophages (Raw 264.7) treated with the pl4 was examined. The production of NO by macrophages is used as a parameter indicative of anti-allergic activity in terms of the activity of immune cells. For this reason, Raw 264.7 was utilized in producing NO. Raw 264.7 cells were seeded at a density of 5x10^5 cells/well into well plates and treated with LPS (lipopolysaccharide) concurrently with various concentrations of the recombinant protein pl4 (10, 100, 1000 ng/mL). The production amount of NO was measured at different times post-treatment (24H: FIG. 19a, 48H: FIG. 19b, 72H: FIG. 19c). As can be seen in FIGS. 19a to 19c, the production amount of NO was found to peak in the group treated with the His tag-removed pl4. Singularly, pure pl4 (pl4-E) free of the His tag induced the production of NO at a higher level than did pl4 conjugated with
the His tag. This result demonstrates that pure pl4 free of His tag induces anti-allergic activity in mouse macrophages (Raw 264.7).

[154] C. Assay of pl4 for Anti-Allergic Activity in NC/Nga Mice

[155] For use in an in vivo assay for confirming the anti-allergic activity of the lactic acid bacteria-derived pl4 of the present invention, the hair of NC/Nga mice, which are an animal model for atopic dermatitis, was cut off from the neck down to the tail using hairclippers and a depilatory. For one day after shaving, the mice were left lest they might be affected by skin diseases and injuries which would be generated during the shaving procedure, other than atopic dermatitis. Each mouse was challenged eight times in total with 200 µL of a solution of 1% DNCB in a mixture of 1:3 acetone : olive oil to induce atopic dermatitis.

[156] After the induction of atopic dermatitis, pl3 or pl4 was intraperitoneally injected at a dose of 400 µg into the NC/Nga mice twice a week for four weeks. A sham group was used as a negative control (NC) while for a positive control (PC), NC/Nga mice were treated with DNCB, but without the protein. During the experiment for four weeks, ocular blood was taken every week from the mice.

[157] Four weeks later, the mice were sacrificed and the affected areas were shaved to examine the progress of atopic dermatitis with the naked eye. The atopic dermatitis had been improved in both the pl3- and the pl4-administered group, compared to the positive group (FIG. 20). In addition, the mouth and the area where the front legs could not reach (the front legs, lateral sides and tail) were in a better condition in the pl4-administered group than the pl3-administered group. pl4 was found to be have a greater therapeutic effect on atopic dermatitis than pl3, as observed with the naked eye. The secondary inflammation caused by scratching due to itching was significantly reduced by the administration of pl4.

[158] Many international scientific journals had it that the level of immunoglobulin E (IgE) is exacerbated in patients with atopic dermatitis, compared to healthy persons. In practical clinics, agents for reducing IgE levels are used. Hence, IgE is known as an index for allergy therapy. Cytokines secreted from Th2, such as IL-4, IL-5 and IL-13, are reported to stay at higher levels in atopic dermatitis patients than healthy persons. Together with IgE, IL-4, representative of the cytokines of Th2, is used as a main index for atopic dermatitis. Serum IgE levels were measured by enzyme-linked immunoserological assay (ELISA). Serum IgE levels were found to significantly decrease in both the pl3- and the pl4-administered group, compared to the positive control (FIG. 21), as measured by ELISA.

[159] Serum IL-4 levels were compared in the same manner as described above. Both the pl3- and pl4-administered group had lower serum IL-4 levels, compared to the
positive control (FIG. 22). Particularly, the serum IL-4 level in the pl4-administered group was lower than that in the pl3-administered group. Taken together, the data obtained above demonstrate that the protein pl4 of the present invention has therapeutic activity for atopic dermatitis in vivo.

[161] Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

[163]
Claims

[Claim 1] Protein pl4, represented by an amino acid sequence of SEQ ID NO: 1 or 3, having anticancer activity.

[Claim 2] The anticancer protein pl4 of claim 1, being derived from *Lactobacillus casei*, *Lactobacillus paracasei* or *Lactobacillus rhamnosus*.

[Claim 3] The protein pl4 of claim 1, having a molecular weight of approximately 14kDa.


[Claim 5] The gene of claim 4, having a nucleotide sequence of SEQ ID NO: 2 or 4.


[Claim 8] An anticancer pharmaceutical composition, comprising protein pl4 as an active ingredient as an active ingredient, said protein pl4 having an amino acid sequence of SEQ ID NO: 1 or 3.

[Claim 9] The anticancer pharmaceutical composition of claim 8, wherein the protein is derived from *Lactobacillus casei*, *Lactobacillus paracasei* or *Lactobacillus rhamnosus*.

[Claim 10] The anticancer pharmaceutical composition of claim 8, wherein the protein has a molecular weight of approximately 14 kDa.

[Claim 11] The anticancer pharmaceutical composition of any one of claims 8 to 10, further comprising a pharmaceutically acceptable excipient, diluent, carrier or buffer.

[Claim 13] An anticancer pharmaceutical composition, comprising the gene of claim 4 as an active ingredient.

[Claim 14] The anticancer pharmaceutical composition of claim 13, wherein the gene has a nucleotide sequence of SEQ ID NO: 2 or 4.

[Claim 15] The anticancer pharmaceutical composition of claim 14, wherein the gene is carried by an expression vector.

[Claim 16] The anticancer pharmaceutical composition of claim 15, wherein the expression vector is a plasmid or a viral vector.

[Claim 17] Protein pl4, represented by an amino acid sequence of SEQ ID NO: 1 or 3, having an anti-allergic activity.

[Claim 18] The protein pl4 of claim 17, being derived from *Lactobacillus casei*, *Lactobacillus paracasei* or *Lactobacillus rhamnosus*.

[Claim 19] The protein pl4 of claim 17, having a molecular weight of approximately 14kDa.


[Claim 21] The gene of claim 4, having a nucleotide sequence of SEQ ID NO: 2 or 4.

[Claim 22] An anti-allergy pharmaceutical composition, comprising:
(a) the protein pl4 of any one of claims 8 to 10, having an anti-allergic activity; and
(b) a pharmaceutically acceptable excipient, diluent, carrier or buffer.
[Fig. 3]

M 16 17 18 19

[Fig. 4]

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<tr>
<th>Reference protein</th>
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<th>Score XC</th>
<th>Molecular weight (Da)</th>
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<tr>
<td>Cell wall-associated hydrolase</td>
<td></td>
<td></td>
<td></td>
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<td>(Lactobacillus casei ATCC 334)</td>
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RECTIFIED SHEET (RULE 91) ISA/KR
[Fig. 6]

Lane 1: clone No. 7 (non-cut)
Lane 2: clone No. 10 (non-cut)
Lane 3: clone No. 4 (cutted)
Lane 4: clone No. 5 (cutted)
Lane 5: clone No. 6 (cutted)
Lane 6: clone No. 7 (cutted)
Lane 7: clone No. 10 (cutted)

[Fig. 7]
[Fig. 8]

Time course analysis

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<tr>
<th>IPTG (mM)</th>
<th>0h</th>
<th>1h</th>
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<tr>
<td>0.005</td>
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<td>0.05</td>
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</table>

13 kDa

14 kDa

49 kDa
[Fig. 10]

p13

\[
\begin{array}{ccccccc}
30 & 40 & 50 & 60 & 70 \\
\end{array}
\]

p13

(1) AKDVPI TTAGGTS DGI G T D LDIQ: AIGDN PASELLVF YD LG SAKM NL D I

p13 QTOP-1

(1) --DVPI TTAGGTS DGI G T D LDIQ: ------------------------------

p13 QTOP-2

(1) ------------------------------: AIGDN PASELLVF YD LG SAKM ----

Consensus

(1) DVPI TTAGGTS DGI G T D LDIQKA IGDNPASELLVF YD LG SAKM

p14

\[
\begin{array}{ccccccc}
80 & 90 & 100 & 110 & 123 \\
\end{array}
\]

p14

(1) DYENGRIANIMWDMDK IKTREEAMKILA DELGLTVPKIVMRKIDEQVF

p14 QTOP-1

(1) --IANIMWDMDK-----------------------------------------------

p14 QTOP-2

(1) ------------------------------KILA DELGLTVPKI----------------

p14 QTOP-3

(1) ----RIANIMWDM DK IK T ---------------------------------------

Consensus

(1) RIANIMWDM DK IK T     KILA DELGLTVPKI

[Fig. 11a]

![Graph showing cell survival percentage for p13 and p14 with ng levels at 0, 10, 100, 1000]
[Fig. 14]

**Daily Feed Intake (g)**

<table>
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<tr>
<th>sham</th>
<th>PC</th>
<th>p13</th>
<th>p14</th>
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</thead>
<tbody>
<tr>
<td>12.5</td>
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[Fig. 15a]

**ACF count**

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<th>sham</th>
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INTERNATIONAL SEARCH REPORT

International application No. PCT/KR20 12/006906

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/335(2006.01)i, A61K 38/16(2006.01)i, A61K 35/74(2006.01)i, A61P 37/08(2006.01)i, A61P 35/00(2006.01)i

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)

C07K 14/335; C07K 14/00; C07K 14/415

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: Protein p14, lactobacillus, cancer, tumor, allergy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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☐ Further documents are listed in the continuation of Box C. ☒ 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
  "E" earlier application or patent but published on or after the international filing date
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  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search
12 FEBRUARY 2013 (12.02.2013)

Date of mailing of the international search report
13 FEBRUARY 2013 (13.02.2013)

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Facsimile No. 82-42-472-7140

Authorized officer
KIM, YUN-KYUNG
Telephone No. 82-42-481-5605

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