

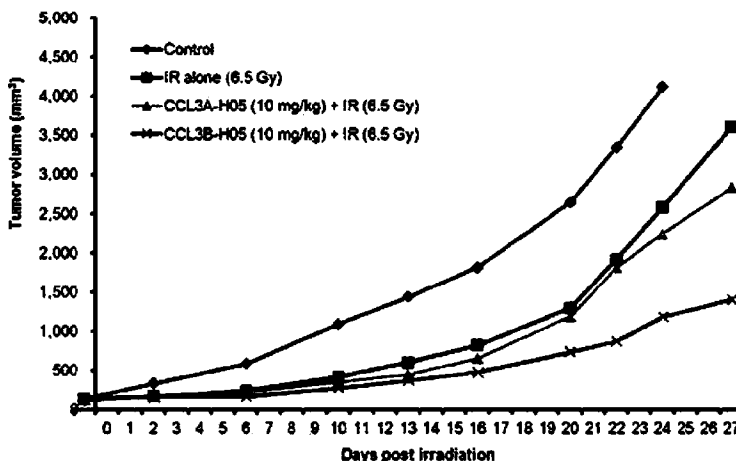


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 (54) Title: FUSION PROTEIN COMPRISING CCL3 VARIANT AND USE THEREOF CROSS-REFERENCE TO RELATED
 APPLICATIONS

**Anti-tumor efficacy of CCL3B-H05
 following intravenous administration to mice
 (BNL 1ME A.7R.1 model, BALB/c mice)**



(57) Abrégé/Abstract:

Provided are a fusion protein having a CCL3 variant with improved in vivo persistency, protein stability and pharmacological activity and a use thereof, more particularly, a fusion protein comprising a CCL3 variant and an immunoglobulin Fc region and a use

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(57) **Abrégé(suite)/Abstract(continued)**:

thereof as a therapeutic agent for lymphopenia, cancer or infection, in which an N-terminal amino acid of a wild-type CCL3 α or CCL3 β is deleted and an amino acid at a specific position is substituted with a different amino acid at the same position of the wild-type CCL3 α or CCL3 β in the CCL3 variant.

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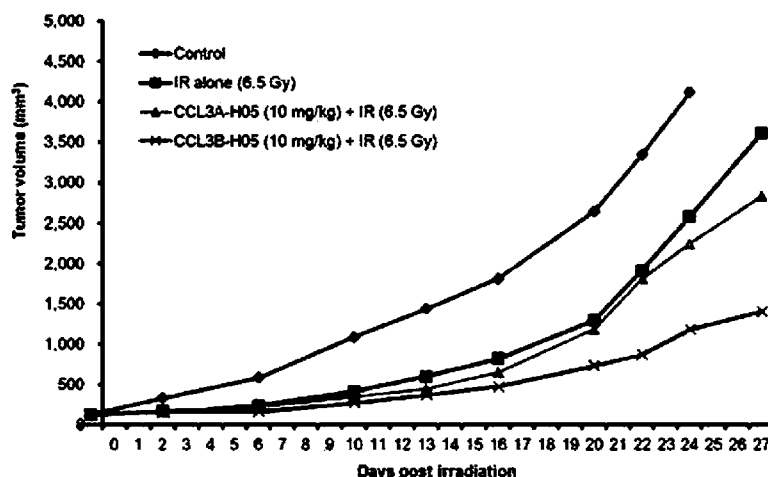
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(54) Title: FUSION PROTEIN COMPRISING CCL3 VARIANT AND USE THEREOF CROSS-REFERENCE TO RELATED APPLICATIONS

Anti-tumor efficacy of CCL3B-H05 following intravenous administration to mice (BNL 1ME A.7R.1 model, BALB/c mice)



(57) Abstract: Provided are a fusion protein having a CCL3 variant with improved *in vivo* persistency, protein stability and pharmacological activity and a use thereof, more particularly, a fusion protein comprising a CCL3 variant and an immunoglobulin Fc region and a use thereof as a therapeutic agent for lymphopenia, cancer or infection, in which an N-terminal amino acid of a wild-type CCL3 α or CCL3 β is deleted and an amino acid at a specific position is substituted with a different amino acid at the same position of the wild-type CCL3 α or CCL3 β in the CCL3 variant.

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WO 2017/188653 A1

WO 2017/188653 A1 

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Description

Title of Invention: FUSION PROTEIN COMPRISING CCL3 VARIANT AND USE THEREOF CROSS-REFERENCE TO RELATED APPLICATIONS

Technical Field

[1] The present invention relates to a fusion protein comprising a CCL3 variant with improved *in vivo* persistency, protein stability, and pharmacological activity, more particularly, to a fusion protein comprising a CCL3 variant and an immunoglobulin Fc region, and a use thereof as a therapeutic agent for lymphopenia, cancer or infection, in which an N-terminal amino acid of a wild-type CCL3 α or CCL3 β is deleted and an amino acid at a specific position is substituted with a different amino acid at the same position of the wild-type CCL3 α or CCL3 β in the CCL3 variant.

[2]

[3] Description of the Related Art

[4] Chemokines are secreted by various immune cells as cytokines having chemotaxis and bind to chemokine receptors expressed in the immune cells to promote *in vivo* mobility of the immune cells. Among the chemokines, CC type ligand 3 (CCL3) is a chemokine that is secreted in immune cells including macrophages and known to be involved in mobility of immune cells expressing a CC type receptor CCR1/CCR5, such as dendritic cells, T cells, monocytes, and neutrophils (Cytokine & Growth Factor Reviews (2002) 13:455-481).

[5] CCL3, known as macrophage inflammatory protein-1 α (MIP-1 α), has only one subtype in mouse, but two subtypes in human (Cytokine & Growth Factor Reviews (2002) 13:455-481). Accordingly, CCL3 includes an α type (LD78 α or CCL3 α , hereinafter, CCL3 α) having homology with that of mouse and a β type (LD78 β or CCL3 β , hereinafter, CCL3 β) generated by mutation of genes in evolutionary process.

[6] In the related art, it is known that CCL3 inhibits the division and differentiation of immature blood stem cells in the bone marrow of mouse and may increase the concentration of immune cells in the blood (Nat Rev Immunol.(2006) 6(2):159-64). As a result, CCL3 has been used as a therapeutic agent target for treatment of lymphopenia that is found in various anticancer agents (Exp. Hematol. (1999) 27:195-202, Cytokine & Growth Factor Reviews (2002) 13:455-481). In addition, since CCL3 has an effect of increasing the concentration of dendritic cells in the blood, it is known that the CCL3 may have potential as a novel immune cancer agent when combined with a treatment method capable of exposing a tumor-specific antigen in the blood (Clin Cancer Res. (2008) 14(4):1159-1166, European Journal of Cancer (1998)

34(7):1023-1029).

- [7] Meanwhile, since CCL3 has a very short half-life and has a tendency to be precipitated easily, CCL3 is not suitable to be used as a biotherapeutic agent without modification. Because the *in vivo* half-life of CCL3 intravenously injected to human body is as short as about 1.7 hours or less, if CCL3 is developed as an anti-cancer agent or a therapeutic agent for lymphopenia, the agent needs to be administered daily (European Journal of Cancer (1998) 34(7):1023-1029). Further, CCL3 is precipitated by the formation of a polymeric substance even at a concentration as low as 0.1mg/mL.
- [8] Therefore, in order to develop CCL3 as a drug or an immune anticancer agent, a CCL3 variant (CCL3 variant) may be prepared by substituting or removing some amino acids of wild-type CCL3, and/or a fusion protein may be prepared by combining a polymer or an additional protein with CCL3. It is known that the half-life may be increased by combining a polymer or an additional protein. For example, an active protein may be bound to human albumin or polyethylene glycol (PEG). However, the residence time is increased only slightly by the binding of human albumin, and the receptor binding affinity may be reduced due to steric hindrance in the binding of PEG.
- [9] Considering this, research and development has been recently performed to increase the *in vivo* half-life through a fusion protein prepared by using immunoglobulin (Ig). Human Ig (hIg) includes various classes, such as IgG, IgM, IgA, IgD, and IgE, and may be further classified into various subtypes known as human IgG1 (hIgG1), human IgG2 (hIgG2), human IgG3 (hIgG3), and human IgG4 (hIgG4).
- [10] Immunoglobulin includes four polypeptide chains; two heavy chains and two light chains are linked through disulfide bonds to form a tetramer. Each chain includes a variable region and a constant region. A constant region of a heavy chain is further classified into three parts, which are CH1, CH2, and CH3, or four parts, which are CH1, CH2, CH3, and CH4, according to isotypes, and includes a hinge, CH2, CH3 and/or CH4 domains.
- [11] Under the technical background, the inventors of the present invention verified that the *in vivo* half-life was increased, the stability was improved, and the drug efficacy was improved in the fusion protein comprising a CCL3 variant and an immunoglobulin Fc region, in which an N-terminal amino acid of a wild-type CCL3 α or CCL3 β is deleted and an amino acid at a specific position is substituted with a different amino acid at the same position of the wild-type CCL3 α or CCL3 β in the CCL3 variant.

[12]

[13] **Summary**

- [14] The present invention provides a fusion protein comprising a CCL3 variant and an immunoglobulin Fc region, in which some amino acids of a wild type CCL3 are substituted and deleted in the CCL3 variant.

- [15] The present invention provides a nucleic acid encoding the fusion protein, a vector comprising the nucleic acid, and cells transformed by using the vector.
- [16] The present invention provides a method of preparing the fusion protein.
- [17] The present invention provides a pharmaceutical composition comprising the fusion protein.
- [18] The present invention provides a CCL3 β variant, in which some amino acids of a wild type CCL3 are substituted and deleted in the CCL3 β variant.
- [19] The present invention provides a fusion protein comprising a CCL3 variant comprising the following mutations and an immunoglobulin Fc region, in which the mutations include: (1) deletion of one or two amino acids from an N-terminal of a wild-type CCL3; and (2) substitution of aspartic acid at the position 27th amino acid from the N-terminal of the wild-type CCL3 with alanine.
- [20] The present invention provides a nucleic acid encoding the fusion protein.
- [21] The present invention provides a vector comprising the nucleic acid.
- [22] The present invention provides cells transformed with the vector.
- [23] The present invention provides a method for preparing the fusion protein.
- [24] The present invention provides a pharmaceutical composition comprising the fusion protein.
- [25] The present invention provides a CC type ligand 3 β (CCL3 β) variant comprising the following mutations: (1) deletion of one or two amino acids from an N-terminal of a wild-type CCL3 β ; and (2) substitution of aspartic acid at the position 27th amino acid from the N-terminal of the wild-type CCL3 with alanine.
- [26] The fusion protein of the present invention comprising the CCL3 variant has a desired pharmacokinetic profile because the activity reduction of the CCL3 variant is minimized, the risk of immunogenicity is low, and the *in vivo* half-life is increased without a stability problem, thereby improving *in vivo* persistency, physical properties and pharmacological efficacy of the protein. The pharmaceutical composition comprising the fusion protein comprising the CCL3 variant can be used as a therapeutic agent for lymphopenia, cancer or infection.

[27]

[28] **Brief Description of The Drawings**

[29] FIG. 1 is a plot showing the results of measuring *in vitro* chemotaxis of CCL3 variant fusion proteins CCL3B-H05 and CCL3B-H40 by using a THP-1 cell line.

[30] FIG. 2 is a plot showing the results of measuring *in vitro* chemotaxis of CCL3 and CCL3 variant fusion proteins CCL3A-H05 and CCL3B-H05 by using a THP-1 cell line.

[31] FIG. 3 shows a result of measuring *in vitro* activity of CCL3 and CCL3 variant fusion proteins CCL3A-H05 and CCL3B-H05 by using a cell line in which CCR1 is

expressed, in order to evaluate reactivity of CCR1 receptors of the fusion proteins.

[32] FIG. 4 shows a result of calculating a pharmacokinetic parameter by measuring a blood concentration of CCL3B-H05 in a blood sample until 144 hours after intravenously injecting the CCL3 variant fusion protein CCL3B-H05 to mice at doses of 3 mg/kg and 10 mg/kg, respectively.

[33] FIG. 5 shows the effect of the tumor growth inhibition of the CCL3 variant fusion protein CCL3B-H05 on a mouse liver cancer.

[34] FIG. 6 shows a drug treatment schedule in Experimental Example 4.

[35]

[36] **Detailed Description of The Preferred Embodiment**

[37] Unless otherwise defined, all technical and scientific terms used in this specification have the same meaning as those commonly understood by those skilled in the art. In general, the nomenclature used in this specification is well-known and commonly used in the art.

[38] Among various long-acting technologies for increasing the half-life of a general protein, an Fc fusion technology is most widely used because the technology may increase the *in vivo* half-life with less concern about side effects such as toxicity or induction of an immune response. Development of Fc fusion CCL3 and/or its variant as a drug for continuous treatment should satisfy several conditions as follows.

[39] First, the decrease of *in vitro* activity by fusion needs to be small. In general, when small proteins such as chemokines are fused with Fc having a relatively large size, it is known that the activity is greatly dependent on a fusion location and a linker. Accordingly, the activity of CCL3 and/or its variant and the Fc fusion protein may vary according to the presence of fusion or a fusion location.

[40] Second, considering that most biopharmaceuticals may cause immunogenicity in patients, the risk of immunogenicity by fusion linkers or mutations needs to be low.

[41] Third, there should be no stability problems due to a fusion location or introduction of mutation.

[42] Fourth, because an undesired immune response may be caused depending on an isotype of fused immunoglobulin, an alternative of the isotype of fused immunoglobulin is required.

[43] While the inventors of the present invention made an effort to improve physiological activities and physical properties of CCL3 by considering the abovementioned conditions, it was found that artificially removing an N-terminal amino acid, introducing a mutation to a specific position of CCL3, and fusion to an immunoglobulin Fc region may increase the activity of the CCL3 to increase the *vivo* exposure degree and half-life and to improve the pharmacological efficacy.

[44] Based thereon, one aspect of the present invention relates to a fusion protein

comprising a CC type ligand 3 (CCL3) variant comprising the following mutations and an immunoglobulin Fc region, wherein the mutations are: (1) deletion of one or two amino acids from an N-terminal of wild-type CCL3; and (2) substitution of aspartic acid at the position 27th amino acid from the N-terminal of the wild-type CCL3 with alanine.

- [45] The CCL3 is a chemokine known to play an important role in mobility homeostasis of dendritic cells and immune cells such as T cells, monocytes, and neutrophils, and may be derived from mammals such as humans, mice, pigs, and monkeys. For example, two subtypes exist in humans. The two subtypes are an α type (hereinafter, referred to as CCL3 α) having homology with that of mouse and a β (hereinafter, referred to as CCL3 β) generated by mutation of genes in evolutionary process. Particularly, the wild-type CCL3 may be, for example, a human wild-type CCL3 α protein comprising a sequence of SEQ ID NO: 1 or a human wild-type CCL3 β protein comprising a sequence of SEQ ID NO: 3.
- [46] In one example, the CCL3 variant may include a CCL3 α variant, in which the CCL3 α variant is prepared by deleting alanine that is the first amino acid from an N-terminal and by substituting aspartic acid that is the 27th amino acid from the N-terminal with alanine in the human wild-type CCL3 α comprising the sequence of SEQ ID NO: 1. The CCL3 α variant may include for example, a sequence of SEQ ID NO: 2.
- [47] In another example, the CCL3 variant may include a CCL3 β variant, in which the CCL3 β variant is prepared by deleting alanine and proline (AP) that are two amino acids from an N-terminal and by substituting aspartic acid that is the 27th amino acid from the N-terminal with alanine in the human wild-type CCL3 β comprising the sequence of SEQ ID NO: 3. The CCL3 β variant may include, for example, a sequence of SEQ ID NO: 4.
- [48] The term "Fc region" herein means a protein without variable regions in heavy and light chains and constant region 1 in light chain (CL1) of an immunoglobulin, and the Fc region may be a hybrid Fc comprising at least one Fc region selected from the group consisting of IgG1, IgG2, IgG3, IgG4, and IgD and fragments thereof or a combination thereof.
- [49] In one example, for example the hybrid Fc may include an IgG4 region and an IgD region. Further, the hybrid Fc region may include part of the hinge sequence and CH2 of an IgD Fc, and CH2 and CH3 sequences of IgG4 Fc, for example, a sequence of SEQ ID NO: 14. The hybrid Fc may be equal to, for example, a hybrid Fc form disclosed in Korean Patent Registration No. 0897938, and introduced to this specification as a reference.
- [50] In yet another example, the Fc region may be a hybrid Fc comprising at least one Fc region selected from the group consisting of IgG1, IgG2, IgG3, and IgG4, and

fragments thereof or a combination thereof.

- [51] The Fc region may include the entire Fc region constituting an immunoglobulin or may include a fragment or an Fc region variant thereof. The Fc region may also include an Fc region variant prepared by substituting some amino acids or combining different types of Fc regions. The Fc region variant may be modified for preventing cleavage at the hinge region. In addition, a part of the amino acid sequence of a hinge sequence of the Fc may be substituted in order to reduce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Further, in the hinge sequence of the Fc, a part of the amino acid sequence may be substituted in order to inhibit rearrangement of a Fab region. Furthermore, lysine (K) at the Fc region C-terminal may be removed.
- [52] Further, the Fc fragment of the present invention may be a wild-type sugar chain, an increased sugar chain compared to the wild type, a decreased sugar chain compared to the wild type, or a form in which the sugar chain is removed. The increase, decrease, or removal of the sugar chain may be performed by a general method known in this art, such as a chemical method, an enzymatic method, and a genetic engineering method using microorganisms.
- [53] The immunoglobulin Fc region may be a form in which the CCL3 variant is directly linked to an N-terminal or a C-terminal of the Fc region or linked to an N-terminal or a C-terminal of the Fc region through a linker. When the immunoglobulin Fc region is directly linked to the CCL3 variant, for example, in the present invention, the CCL3 α variant of SEQ ID NO: 2 or the CCL3 β variant of SEQ ID NO: 4 may be linked to an N-terminal or a C-terminal of the Fc region of SEQ ID NO: 14. The linked form of the CCL3 and the Fc may appropriately be a form in which the CCL3 is linked to an N-terminal of the Fc region.
- [54] When the immunoglobulin Fc region is linked through a linker, the linker may be linked to an N-terminal, a C-terminal, or a free radical of the Fc fragment and the may be linked to an N-terminal, a C-terminal, or a free radical of the CCL3 variant. When the linker is a peptide linker, the linkage may occur at any part. For example, the linker may be linked to a C-terminal of the Fc region of the immunoglobulin and an N-terminal of the CCL3 variant.
- [55] After a linker and the Fc are separately expressed and then linked to each other, the linker may be a crosslinker known in this art. The crosslinker may be, for example, N-hydroxysuccinimide ester such as 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, and 4-azidosalicylic acid, imidoesters comprising disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane, but is not limited thereto.
- [56] In addition, the linker may be a peptide. Particularly, the linker may be a peptide

consisting of 10 to 30 amino acid residues. In one example, the immunoglobulin Fc region may be linked to the CCL3 variant through the linker as set forth in Formula 1 below.

[57]

[58] (Formula 1)

[59] $(RNT)_nGRGG(EEKKK)_m$

[60]

[61] In Formula 1, n and m are 1 to 5, respectively. In Formula 1, n represents the repetition number of arginine-asparagine-threonine amino acids, m represents the repetition number of glutamate-glutamate-lysine-lysine-lysine amino acids, and preferably, n and m may be 1 to 3, respectively.

[62] In one example, by considering that n and m may be 1 to 3, respectively, the linker may include any one sequence selected from the group consisting of SEQ ID NOs: 5 to 13.

[63] One specific example of the present invention includes a structure in which the immunoglobulin Fc region is linked to the CCL3 variant through the linker comprising the sequence of RNTGRGGEEKKK (SEQ ID NO: 5). One Experimental Example of the present invention showed that the CCL3 variant fusion protein bound to the immunoglobulin Fc through the linker comprising the sequence of SEQ ID NO: 5 had excellent chemotaxis activity.

[64] The fusion protein may be a form in which a dimer or multimer CCL3 variant is bound to the Fc region of the immunoglobulin, in which the dimer or multimer CCL3 variant is formed when one or more CCL3 variants are bound with each other. In addition, the fusion protein may be a dimer or multimer form, in which the dimer or multimer form may be two or more Fc regions are linked, wherein the immunoglobulin Fc regions have the CCL3 variant connected thereto.

[65] Another aspect of the present invention relates to a nucleic acid encoding the fusion protein comprising the CCL3 variant.

[66] The nucleic acid encoding the fusion protein comprising the CCL3 variant may be isolated to recombinantly produce the fusion protein comprising the CCL3 variant. The nucleic acid is isolated and inserted into a replicable vector to perform an additional cloning or expression. Based thereon, another aspect of the present invention relates to a vector comprising the nucleic acid.

[67] The term "nucleic acid" used herein has a comprehensive meaning comprising DNA (gDNA and cDNA) and RNA molecules, and a nucleotide as a basic building block of a nucleic acid includes not only a natural nucleotide but also analogues thereof comprising a modified sugar or base site. A sequence of the nucleic acid encoding the fusion protein comprising the CCL3 variant of the present invention may be modified.

The modification includes addition, deletion, or non-conservative substitution or conservative substitution of a nucleotide.

[68] In the present invention, the term “nucleic acid” includes a nucleotide sequence having substantial identity with the respect to the sequence. The substantial identity means an amino acid sequence having at least 80% homology, more preferably at least 90% homology, and most preferably at least 95% homology, in an analysis performed by aligning the nucleic acid sequence of the present invention to correspond to an another arbitrary sequence as much as possible and then analyzing the aligned sequence by using algorithms commonly used in the art.

[69] A DNA encoding the fusion protein comprising the CCL3 variant is easily isolated or synthesized by using a process commonly used in this art.

[70] The term “vector” used herein refers to a means for expressing a target gene in a host cell, and includes plasmid vectors; cosmide vectors; virus vectors such as bacteriophage vectors, adenovirus vectors, retrovirus vectors, and adeno-associated virus vectors; and the like. Acceptable vector components generally include one or more of a signal sequence, a replication origin, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, but are not limited thereto.

[71] In the vector, the nucleic acid encoding the fusion protein comprising the CCL3 variant is operatively linked to the promoter.

[72] The term “operatively linked” used herein means a functional binding between a nucleic acid expression regulatory sequence (for example, a promoter, a signal sequence, or an array at a binding site of a transcription regulator) and a different nucleic sequence, and the regulatory sequence regulates through the functional binding the transcription and/or translation of the different nucleic sequence.

[73] In the case of using prokaryotic cells as a host, generally, a strong promoter (for example, a tac promoter, a lac promoter, a lacUV5 promoter, a lpp promoter, a pL λ promoter, a rac5 promoter, an amp promoter, a recA promoter, a SP6 promoter, a trp promoter, a T7 promoter, and the like), a ribosome binding site for initiation of translation, and a transcription/translation termination sequence are generally included. In addition, for example, in the case of using eukaryotic cells as a host, promoters derived from a genome of mammalian cells (for example, a metallothionein promoter, an β -actin promoter, a human hepatoblast promoter and a human muscle creatine promoter) or promoters derived from mammalian viruses (for example, an adenovirus late-phase promoter, a vaccinia virus 7.5K promoter, a SV40 promoter, a cytomegalovirus (CMV) promoter, an HSV tk promoter, a mouse breast tumor virus (MMTV) promoter, an LTR promoter of HIV, a moloney promoter, an Epstein-Barr virus (EBV) promoter, and a promoter of rosacekoma virus (RSV)) may be used, and a polyadenylation sequence is generally included as a transcription termination

sequence.

- [74] In some cases, the vector may be fused with a different sequence in order to facilitate purification of the fusion protein comprising the CCL3 variant expressed therefrom. The fused sequence includes, for example, glutathione S-transferase (Pharmacia, USA), maltose binding protein (NEB, USA), FLAG (IBI, USA), 6x His (hexahistidine; Quiagen, USA), and the like.
- [75] The vector includes as a selectable marker an antibiotic resistance gene commonly used in this art, such as genes having resistance to ampicillin, gentamycin, carbenicillin, chloramphenicol, streptomycin, kanamycin, geneticin, neomycin and tetracycline.
- [76] Another aspect of the present invention relates to cells transformed by the aforementioned vector. The cells may be prokaryotes, yeast or higher eukaryotic cells, but are not limited thereto.
- [77] *Escherichia coli*, bacillus species strains, such as *bacillus subtilis* and *bacillus tulignensis*, and prokaryotic host cells, such as *streptomyces*, *pseudomonas* (for example, *pseudomonas putida*), *proteus mirabilis*, and *staphylococcus* (for example, *staphylococcus carnosus*), may be used.
- [78] However, interest in animal cells is greatest, and an example of a useful host cell line may be COS-7, BHK, CHO, CHOK1, DXB-11, DG-44, CHO/-DHFR, CV1, COS-7, HEK293, BHK, TM4, VERO, HELA, MDCK, BRL 3A, W138, Hep G2, SK-Hep, MMT, TRI, MRC 5, FS4, 3T3, RIN, A549, PC12, K562, PER.C6, SP2/0, NS-0, U20S, or HT1080, but is not limited thereto.
- [79] Another aspect of the present invention relates to a method of preparing a fusion protein comprising a CCL3 variant, in which the method includes (a) culturing the cells; and (b) collecting the fusion protein in the cultured cells.
- [80] The cells may be cultured in various media. Commercially available media may be used as the culture medium without limitation. All other essential supplements known to those skilled in this art may be included at an appropriate concentration. It is obvious for the one skilled in the art to select optimal culturing conditions, for example, temperature, pH, and the like in culturing the host cells.
- [81] The recovery of the fusion protein comprising the CCL3 variant may be performed by removing impurities by, for example, centrifugation or ultrafiltration, and purifying the resulting product by using, for example, affinity chromatography and the like. Other additional purification techniques, for example, anion or cation exchange chromatography, hydrophobic interaction chromatography, hydroxylapatite chromatography, and the like may be used.
- [82] Another aspect of the present invention relates to a pharmaceutical composition comprising the fusion protein comprising the CCL3 variant.

- [83] The pharmaceutical composition may include a fusion protein comprising a CCL3 variant at an effective amount for treatment and a pharmaceutically acceptable carrier. The term “pharmaceutically acceptable carrier” refers to a material that may be added to an active component to help the formulating or stabilizing the formulation, and does not cause a significantly harmful toxic effect on a patient.
- [84] The carrier means a carrier or a diluent neither irritating a patient and nor inhibiting biological activity and characteristics. The pharmaceutical carrier acceptable for a composition formulated into a liquid solution is suitable for sterilization and bio-compatible. A saline solution, sterile water, a ringer's solution, buffered saline, an albumin injection solution, a dextrose solution, a maltodextrin solution, glycerol, ethanol, and a mixture of one or more thereof may be used, and if necessary, other general additives such as antioxidants, buffers, and bacteriostats may be added. In addition, the pharmaceutical carrier may be formulated as injectable solutions, such as aqueous solutions, suspensions, emulsions, and the like, pills, capsules, granules or tablets by additionally adding diluents, dispersants, surfactants, binders and lubricants.
- [85] The pharmaceutically acceptable carrier includes a sterile aqueous solution or a dispersion and sterile powder for extemporaneously preparing a sterile injectable solution or dispersion. Use of such a medium and an agent for a pharmaceutically active substance is known in this art. The composition is preferably formulated for parenteral injection. The composition may be formulated as solutions, microemulsions, liposomes, or other ordered structures suitable for a high drug concentration. The carrier may be a solvent or a dispersive medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), and an appropriate mixture thereof. In some cases, isotonic agents, for example, sugars, polyalcohols such as mannitol and sorbitol, or sodium chloride may be included in the composition. The sterile injectable solution may be prepared by mixing a required amount of active compound in a suitable solvent with one or a combination of the abovementioned components according to the need, and by performing sterile microfiltering of the resulting mixture. Generally, the dispersion agents are prepared by adding an active compound into a sterile vehicle containing a basic dispersion medium and other required components among those described above. Some methods of preparing a sterile injectable solution by using sterile powder include vacuum drying and freeze-drying to produce powder of an active component and any additional desired component from a solution thereof that has already been sterilized and filtered.
- [86] In addition, the present invention relates to a composition for treatment or prevention of CCL3-associated disorders comprising the fusion protein comprising the CCL3 variant or relates to a method for treating or preventing CCL3-associated disorders

comprising administration of the fusion protein comprising the CCL3 variant into a subject requiring treatment.

- [87] The CCL3-associated disorders may include, for example, lymphopenia, various cancers, and infection. The fusion protein comprising the CCL3 variant according to the present invention may induce cancer tissue and/or cancer cell death due to activation of an immune response in association with a cancer cell death caused by irradiation. Based on this, in one example of the present invention, the fusion protein may be a composition of combining or assisting radiation treatment for treatment or prevention of cancer.
- [88] The radiation rays may be, for example, gamma ray or X ray, and is not limited thereto, but may be irradiated at a dose of 0.1 to 50 Gy and, preferably, 0.1 to 10 Gy. The dose of the radiation rays may be adjusted within an appropriate range by considering immune deterioration and the like due to radiation side effects.
- [89] The fusion protein comprising the CCL3 variant may be administered through any routes. For example, the fusion protein comprising the CCL3 variant may be provided to animals directly (for example, by injecting, implanting, or locally administering the fusion protein into a tissue part, locally) or systematically (for example, parenterally or orally) by any appropriate means.
- [90] In the case of parenteral administration, such as intravenous, subcutaneous, ophthalmic, intraperitoneal, intramuscular, oral, rectal, intraorbital, intracerebral, intracranial, intraspinal, intraventricular, intrathecal, intracisternal, intracapsular, intranasal, or aerosol administration, for example, a part of an aqueous or physiologically compatible body-fluid suspension or solution may be included. Accordingly, since the carrier or the vehicle is physiologically acceptable, the carrier or the vehicle may be added to the fusion protein to be transferred to the patient. Therefore, as the carrier such as a body fluid for formulation, generally, a saline solution may be included.
- [91] A dose frequency varies according to pharmacokinetic parameters of the fusion protein comprising the CCL3 variant in the formulation used. Typically, a clinical doctor may administer the fusion protein until reaching a dose achieving a desired effect. Accordingly, the fusion protein may be administered as a single dose, as two or more doses (with or without comprising the same amount of target fusion protein) with a time interval, or as continuous injection through a graft device or a catheter. Additional refinement of the appropriate dose is routinely achieved by those skilled in this art and corresponds to a work field routinely performed by those skilled in this art.
- [92] A unit dose is 0.01 μ g/kg to 100mg/kg, particularly, 1 μ g/weight kg to 30mg/weight kg in humans. The dose is an optimal dose, but may be depending on the diseases to be treated and the existence of side effects, and the optimal dose may be determined by performing experiments commonly carried out. The administration of the fusion

protein may be performed by periodic bolus injections or continuous intravenous, subcutaneous, or intraperitoneal administration from an external reservoir (for example, an intravenous bag) or an internal reservoir (for example, a bioerodable implant).

[93] The administration frequency varies according severity of a disease. The administration frequency may be in a range of three times per week to once per week or once every two weeks.

[94] In some cases, the fusion protein comprising the CCL3 variant may be administered to a target receptor with other biologically active molecules. However, an optimal combination, a dosage form, and an amount of the fusion protein and other molecules may be determined through a common experiment well-known in this art.

[95] The term “effective amount for treatment” used herein refers to a sufficient amount to treat a disease at a reasonable benefit/risk ratio applicable to medical treatment and means an amount of the fusion protein comprising the CCL3 variant according to the present invention. An accurate amount varies according to many factors that include components and physical characteristics of the therapeutic composition, an intended patient population, individual patient considerations, and the like, but are not limited thereto, and may be easily determined by those skilled in this art. When completely considering these factors, it is important to administer a minimum amount sufficient to achieve a maximum effect without side effects and the dose may be easily determined by experts in this art.

[96] The dose of the pharmaceutical composition of the present invention is not particularly limited, but is changed by various factors comprising the health condition and weight of a patient, the severity of the disease, the kind of the drug, the administration route and the administration time. The composition may be administered into mammals comprising rats, mice, livestock, humans, and the like once or multiple times daily through a typically acceptable route, for example, orally, rectally, intravenously, subcutaneously, intrauterinely, or intracerebrovascularily.

[97] Another aspect of the present invention relates to a CC type ligand 3 β (CCL3 β) variant comprising the following mutations: (1) deletion of one or two amino acids from an N-terminal of a wild-type CCL3 β ; and (2) substitution of aspartic acid at the position 27th amino acid from the N-terminal of the wild-type CCL3 with alanine.

[98] In one example of the present invention, the CCL3 β variant may include, for example, a sequence of as set forth in SEQ ID NO: 4. The CCL3 β variant of the present invention equally includes the above mentioned structures and the description for duplicated configurations is equally applied even to the invention of the CCL3 β variant.

[99] Unless otherwise defined in the technical and scientific terms used in the present invention, the present invention has a meaning which is generally understood to those skilled in the art. Further, the repeated description for the same technical configuration

and operation as those in the related art will be omitted.

[100]

[101] Examples

[102] Hereinafter, the present invention will be described in more detail through Examples. However, it is apparent to those skilled in the art that the present invention is not limited to the following Examples, and various modifications and changes may be made within the idea and scope of the present invention.

[103]

[104] Preparation Example 1. Preparation and purification of fusion protein comprising CCL3 variant

[105] A mutation study on CCL3 was conducted to improve physical properties and activity profiles of the CCL3 in a CCL3-Fc structure.

[106] Particularly, in order to determine whether the difference of activity between an α type (hereinafter, referred to as CCL3 α) and a β type (hereinafter, referred to as CCL3 β) of the CCL3 protein is maintained even after Fc fusion, various variants were constructed. In addition, in order to inhibit the precipitation of the CCL3 in the Fc fusion protein type, a variant in which the 27th aspartic acid was substituted with alanine was designed.

[107] Locations, sequence information, objects, and expected effects of each mutation introduced into the CCL3 were summarized in Table 1 below.

[108]

[109] [Table 1]

Designation of Sequences	Position	Original Sequence	Mutation Sequence	Object	Expected Effect
D27A	27	D	A	Point mutation	Improvement of physical properties through inhibition of precipitation
$\Delta 1$	1	A	-	Amino acid deletion	Improvement of physical properties
$\Delta 1-2$	1-2	AS or AP	-	Amino acid deletion	Improvement of pharmacological effect
CCL3 α	2, 39, 47	S, G, S	-	-	-
CCL3 β	2, 39, 47	P, S, G	-	-	-

[110]

[111] Amino acids were encoded in an expression vector to express three constituent elements in the order of the CCL3 variant, a linker, and a fusion carrier from an N-terminal to a C-terminal. Table 2 below summarizes symbols of the CCL3 variant fusion proteins, mutation sequences introduced into the CCL3, fusion carrier sequences, and linker sequences.

[112]

[113] [Table 2]

Protein Code	CCL3 mutation sequence	Fusion carrier sequence	Linker sequence
CCL3	D27A, Δ1, CCL3α (SEQ ID No: 2)	N/A	N/A
CCL3A-H05 (SEQ ID No: 15)	D27A, Δ1, CCL3α (SEQ ID No: 2)	hyFc (SEQ ID No: 14)	H05 (SEQ ID No: 5)
CCL3B-H05 (SEQ ID No: 16)	D27A, Δ1-2, CCL3β (SEQ ID No: 4)	hyFc (SEQ ID No: 14)	H05 (SEQ ID No: 5)
CCL3B-H40 (SEQ ID No: 17)	D27A, Δ1-2, CCL3β (SEQ ID No: 4)	hyFc (SEQ ID No: 14)	N/A

[114]

[115] In order to produce the CCL3 variant fusion protein, a nucleotide sequence encoded based on an amino acid sequence of the CCL3 variant fusion protein was synthesized by Bioneer Co., Ltd. (Korea). *NheI* and *NotI* restriction enzyme sequences were added to a 5'-terminus and a 3'-terminus of the nucleotide sequence encoding each CCL3 variant fusion protein, respectively, and a start codon for protein translation and an induction sequence for secreting the expressed protein extracellularly were inserted after the restriction enzyme sequence of the 5'-terminus. A termination codon was inserted after the nucleotide sequence encoding each CCL3 variant fusion protein. A nucleotide sequence encoding each CCL3 variant fusion protein was cloned in a pTrans-empty expression vector by using the two restriction enzyme sequences *NheI* and *NotI*. The pTrans-empty expression vector obtained from CEVEC Corporation in Germany was a simple structural expression vector having a CMV promoter, a pUC-derived replication origin, an SV40-derived replication origin, and an ampicillin resistance gene.

[116]

[117] Preparation Example 1-2. Preparation of plasmid DNA for expression of CCL3 variant fusion protein

[118] A large amount of plasmid DNAs to be used for expression was obtained by transforming each expression vector prepared in Preparation Example 1-1 to *E. coli*. Each expression vector was transduced through heat shock into *E. coli* with a weakened cell wall, and the resulting transduced *E. coli* was smeared on an LB plate to

obtain colonies. The obtained colonies were inoculated into an LB medium and cultured at 37°C for 16 hours, and 100mL of *E. coli* having respective expression vectors in the cells was obtained, respectively. After removing a culture medium through centrifugation, solutions P1, P2, and P3 (QIAGEN, Cat# 12963) were added to the *E. coli* to obtain a DNA turbid solution by breaking the cell wall and isolating the protein and the DNA. The plasmid DNAs were purified from the obtained DNA turbid solution by using a Qiagen DNA purification column. The eluted plasmid DNAs were confirmed by agarose gel electrophoresis and used for expression after measuring a concentration and purity by using a nanodrop device (Thermo scientific, Nanodrop Lite).

[119]

[120] Preparation Example 1-3. Expression of fusion protein in CAP-T cells

[121] A human cell line was transformed with each plasmid DNA isolated in Preparation Example 1-2. Each plasmid DNA was transduced into CAP-T cells (CEVEC) while being cultured in a PEM medium (Life technologies) by using a PEI solution (Polyplus, Cat # 101-10N). A mixed solution of the DNA and the PEI solution was mixed with suspended cells by using a Freestyle 293 expression medium of Invitrogen Corporation, cultured at 37°C for 5 hours, and then added with a PEM medium. After culture at 37°C for 5 to 7 days, the cells were removed by centrifugation to obtain a supernatant comprising the CCL3 variant fusion protein.

[122]

[123] Preparation Example 1-4. Purification of CCL3 variant fusion protein

[124] Impurities were removed from the culture supernatant comprising the CCL3 variant fusion protein by using a 0.2µm filter and then a protein A affinity chromatography column (GE Healthcare) was equilibrated with a 1X PBS (pH 7.4) buffer. The column was washed with the 1X PBS (pH 7.4) buffer and then the protein was eluted with a 100 mM glycine (pH 3.0) buffer. The CCL3A-H05 fusion protein (SEQ ID NO: 15) obtained through the affinity chromatography was purified by using a hydroxyapatite type I column (Ceramic Hydroxyapatite type I, 40µM, Bio-rad), and the CCL3B-H05 fusion protein (SEQ ID NO: 16) and the CCL3B-H40 fusion protein (SEQ ID NO: 17) were purified by using a hydroxyapatite type I column (Ceramic Hydroxyapatite type II, 40µM, Bio-rad). The hydroxyapatite column was equilibrated with a 20mM sodium phosphate (pH 7.2) buffer and then the eluted CCL3 variant fusion protein was loaded on the affinity chromatography. Particularly, the column was washed with the 20 mM sodium phosphate (pH 7.2) buffer, the 20mM sodium phosphate (pH 7.2) buffer flowed at a concentration gradient, and then eluted fractions were analyzed. Each fraction was analyzed by using a size exclusion chromatography (SEC-HPLC) assay, and portions comprising the high-purity CCL3 variant fusion protein were collected

and then dialyzed overnight at 4°C with the final buffer 1X PBS. A protein crude solution obtained by the dialysis was concentrated at 3,000rpm at 4°C by using a 30,000 molecular-weight cutoff centrifugal filter. The concentration of the CCL3 variant fusion protein was measured by a BCA quantitative analysis.

[125]

[126] Experimental Example 1. Result of measuring *in vitro* activity of fusion protein

[127]

[128] Experimental Example 1-1. Result of measuring activity according to linker sequence

[129] The *in vitro* activity of CCL3 variant fusion proteins CCL3B-H05 (SEQ ID NO: 16) and CCL3B-H40 (SEQ ID NO: 17) prepared in the above Preparation Examples was measured.

[130] In particular, in order to evaluate the *in vitro* activity of the fusion proteins, a THP-1 cell line ATCC in which receptors CCR1 and CCR5 of the CCL3s were expressed was used. In order to evaluate the activity, a concentrate comprising the fusion proteins obtained in the Preparation Examples were twice-series diluted and prepared at a concentration of 100µg/mL by using an analysis medium (0.01% BSA in RPMI 1640 Medium), and the THP-1 cell line was transferred to the analysis medium and then diluted at 5,000,000 cells/mL. A polycarbonate transwell system (Costar, Cat # 3422) was used to treat the bottom wells with 600µL of the diluted fusion protein and the top inserts with 100µL of the diluted THP-1 cell line, and then the system was kept in a 5% CO₂ incubator at 37°C for reaction. After 24 hours, the inserts were removed, and the cells were stained with a trypan blue solution (Sigma, Cat # T8154-100ML) and the number of transferred cells was measured by using a cell counter (Invitrogen, Luna cell counter). The activity was compared by calculating an increase in the maximum number of migrated cells in comparison with the number of cells in a control group treated with the analysis medium, and the results are shown in FIG. 1.

[131] As shown in FIG. 1, the result of the measured activity depending on the linkers showed that the chemotaxis of the fusion protein CCL3B-H05 was higher than that of CCL3B-H40.

[132]

[133] Experimental Example 1-2. Result of measuring activity according to CCL3 variant

[134] The *in vitro* activity of the fusion protein CCL3A-H05 of the sequence of SEQ ID NO: 15 and the fusion protein CCL3B-H05 of the sequence of SEQ ID NO: 16 prepared in the above Preparation Examples was measured.

[135] Particularly, in a concentrate comprising the fusion proteins obtained in Preparation Examples, chemotaxis of the fusion proteins were measured by the same method as Experimental Example 1-1, and the results are shown in FIG. 2.

[136]

- [137] Experimental Example 1-3. Result of measuring activity according to CCL3 variant
- [138] The *in vitro* activity of the fusion protein CCL3A-H05 of the sequence of SEQ ID NO: 15 and the fusion protein CCL3B-H05 of the sequence of SEQ ID NO: 16 prepared in the above Preparation Example was measured.
- [139] Particularly, in order to evaluate reactivity to the receptor CCR1 of the fusion protein, a Tango™ CCR1-*bla* U2OS DA cell line in which the receptor CCR1 of the fusion protein was expressed and an analysis kit (Invitrogen, Cat# K1793) were used. For evaluating the activity, the Tango™ CCR1-*bla* U2OS DA cell line was diluted with a basic medium (FreeStyle™ Expression Medium) to the concentration of 312,500 cells/mL, 32μL of the diluted cell line was added to a 384-well plate (Corning, Cat# 3712), and then stored in a 5% CO₂ incubator for 16 to 24 hours. The concentrate comprising the fusion proteins obtained in the Preparation Example was diluted to the concentrations of 10μM for CCL3 and 25μM for CCL3A-H05, which were 5 times higher than the treatment concentration by using the analysis medium (0.5% DMSO in FreeStyle™ Expression Medium). The prepared fusion proteins were added to wells comprising the cells by 8μL to each well to treat the cells with the twice-series diluted fusion proteins at actual concentrations of 2μM for CCL3 and 5μM for CCL3A-H05 and CCL3B-H05, and the treated cells were kept in a 5% CO₂ incubator at 37°C for 5 hours for reaction. A 6X substrate mixed solution (6μL of 1mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60μL of solution B + 904μL of Solution C + 30μL of Solution D) was prepared by using the solution contained in the analysis kit, and then 8μL of the resulting mixed solution was added to each of the wells in the 384-well plate treated with the fusion protein, and then the cells were kept at room temperature for 2 hours for reaction. A ratio (Blue/Green Emission ratio) of the reacted cells and the unreacted cells was measured and calculated by a microplate reader (Molecular devices, Flexstation 3), and the results are shown in FIG. 3.
- [140] As shown in FIG. 3, the reactivity of the CCR1 receptor was at levels of 0.63 nM for CCL3, 16.0 nM for CCL3A-H05, and 11.6 nM for CCL3B-H05.
- [141]
- [142] Experimental Example 2. Evaluation of physical properties of fusion protein
- [143]
- [144] Experimental Example 2-1. Experimental method for evaluating stability
- [145] In order to determine an amount of protein aggregates in an initial state of the sample, the content (% HMW) of high molecular weight aggregates was measured by using a size exclusion chromatography (SEC-HPLC) method.
- [146] Specifically, the SEC-HPLC method was performed by using a Tosoh model TSK-GEL G3000SW_{XL} column. A Buffer 1X PBS was flowed into the column at a flow rate of 1mL/min to equilibrate the column. The CCL3B-H05 protein stock solution

obtained in Preparation Example 1-4 was concentrated at 3000 rpm and 4°C to a target concentration of 9.5mg/mL or higher by using a 30,000 molecular weight cutoff centrifugal filter. The concentration of each sample was measured by BCA quantitative analysis, and then the sample was stored at -70°C for 5 weeks for stability evaluation. In order to measure the high molecular weight aggregate ratio (% HMW), 9.57mg/mL of the sample was diluted with 1X PBS to the concentration of 1 mg/mL, and the high molecular weight aggregate ratio was analyzed by injecting 100µL of the diluted sample into an SEC-HPLC column.

[147] As shown in Table 3, the results verified that %HMW of the CCL3B-H05 was low and the physical property of the CCL3 variant fusion protein was improved without being disturbed by Fc fusion, as a D27A mutant was introduced and thus the %HMW was significantly decreased.

[148]

[149] [Table 3]

Stability (%HMW) of CCL3B-H05 at concentration of 9.5mg/mL

CCL3B-H05 at 0 day	CCL3B-H05 at 5 weeks (37°C)
1.2%	1.0%

[150]

[151] As Table 3 shows, an amount of %HMW at an initial stage (0 day of storage) of storage was not increased but maintained after 5 weeks of storage at -70°C. It is shown that the stability of the fusion protein CCL3B-H05 is maintained regardless of the mutation.

[152]

[153] Experimental Example 3. Pharmacokinetic measurement of fusion protein

[154]

[155] Test Example 3-1. Experimental method for Pharmacokinetic measurement

[156] Seven-week-old male C57BL/6 mice purchased from Orient BIO Corporation in Korea were group-separated (*n*=3 per blood collection time) to have similar average body weights on a day before the drug treatment, and then 3 mg/kg and 10mg/kg of the samples were intravenously administered once, respectively, and the blood samples were collected at 0.083, 0.5, 1, 4, 8, 12, 24, 48, 72, 96 and 144 hours. In order to measure the blood concentration of the fusion protein, a Human CCL3/MIP-1α Quantikine ELISA kit (R&D systems, Cat# SMA00) having immunoreactivity to the CCL3 was used in this experiment. Pharmacokinetic parameters were calculated by measuring the blood concentration of CCL3B-H05 in blood sample up to 144 hours after each fusion protein was intravenously injected into mice.

[157]

[158] Experimental Example 3-2. Result of measuring pharmacokinetic activity
 [159] The pharmacokinetic parameters were calculated based on the blood concentration plot (FIG. 4) over time following the intravenous administration of the fusion protein to the mice, and the calculation results are shown in Table 4 below.

[160]

[161] [Table 4]

Parameters	3mg/kg	10mg/kg
AUC _{last} (μg·hr/mL)	1639403.7	6549244.7
Half-life (Hour)	20.4	45.6
Total clearance (mL/hr/kg)	0.0018	0.0018
Vd _{ss} (mL/kg)	0.0635	0.0636
C ₀ (μg/mL)	46741.6	227907.4

[162]

[163] A pharmacokinetic profile of the fusion protein was compared and evaluated based on area under the curve (AUC), which indicated the degree of exposure to the drug.

[164] As shown in Table 4, when CCL3B-H05 was intravenously administered to the mice at doses of 3 and 10mg/kg, body exposure was generally increased in proportion to the dose, and clearance (CL) and distribution volume (Vd_{ss}) were not significantly dependent on the dose, and thus a linear PK was found in the dose range of 3 to 10mg/kg. The results also showed that a half-life (t_{1/2}) of the CCL3B-H05 was 20.4 hours and 45.6 hours for each dose of 3 and 10mg/kg and increased approximately 12 to 27 times larger than the half-life (within 1.7 hours during intravenous administration in the human) of the CCL3. Considering that the half-life in mice is generally shorter than the half-life in humans, the increase of the half-life of CCL3B-H05 in comparison with the half-life of CCL3 is expected to be larger in mice.

[165]

[166] Experimental Example 4. Evaluation of activity of fusion protein in mice

[167]

[168] Experimental Example 4-1. Experimental method of anticancer efficacy in BNL 1ME A.7R.1 liver cancer allograft mice

[169] Seven-week-old male Balb/c mice purchased from Orient BIO Corporation in Korea were acclimated for one week, and then 1×10⁷ cells of BNL 1ME A.7R.1 (ATCC) which was a mouse liver cancer cell line were injected into the right hindlimb. At the time when a tumor volume was 120 to 150mm³, groups were separated so that the tumor volume became similar. The experimental groups and the drug treatment schedule for each group are shown in Tables 5 and Fig. 6, respectively. From the first

day of drug treatment, the tumor volume was measured by using a caliper every 3 to 4 days to observe the tumor growth inhibitory efficacy of the drug(tumor volume = long axis × (short axis)²/2) .

[170]

[171] [Table 5]

Group	Route of administration		Dosage	Regimen	Number
G1	Control	-	-	-	8
G2	IR	-	6.5 Gy	-	8
G3	IR + CCL3A-H05	IV	6.5 Gy/10mg/kg	q2dx3	8
G4	IR + CCL3B-H05	IV	6.5 Gy/10mg/kg	q2dx3	8

[172]

[173] Experimental Example 4-2. Result of evaluating anticancer efficacy in BNL 1ME A.7R.1 liver cancer allograft mice

[174] The tumor growth inhibitory efficacy of the fusion protein CCL3A-H05 comprising the sequence of SEQ ID NO: 15 and the fusion protein CCL3B-H05 comprising the sequence of SEQ ID NO: 16 prepared in the above Preparation Examples was verified on BNL 1ME A.7R.1 mouse liver cancer. As shown in FIG. 5, the tumor growth inhibitory efficacy was approximately 25% in a group treated with CCL3A-H05 10mg/kg and approximately 72% in a group treated with CCL3B-H05 10mg/kg as compared to a group treated with 6.5 Gy of only single irradiation after one month from the irradiation.

[175] Although the specific part of the present invention has been described in detail, it is obvious to those skilled in the art that such a specific description is just a preferred embodiment and the scope of the present invention is not limited. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

[176]

Sequence Listing Free Text

[177] Attached the electronic file.

Claims

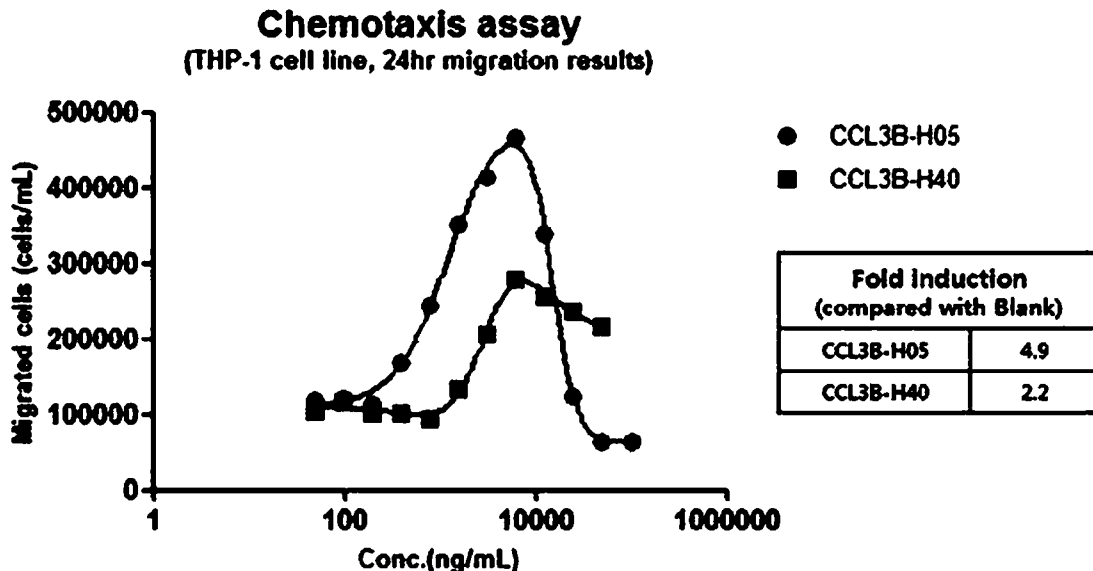
1. A fusion protein comprising a CC type ligand 3 (CCL3) variant comprising the following mutations and an immunoglobulin Fc region; wherein the mutations are
 - (1) deletion of two amino acids, alanine and proline, from the N-terminal of wild-type CCL3 of SEQ ID NO.: 3, and
 - (2) substitution of aspartic acid at the position 27th amino acid from the N-terminal of the wild-type CCL3 of SEQ ID NO.: 3.
2. The fusion protein according to claim 1, wherein the CCL3 variant comprises the sequence of SEQ ID NO: 4.
3. The fusion protein according to claim 1, wherein the CCL3 variant is linked to an immunoglobulin Fc region through a linker presented by Formula 1 below:

(Formula 1)

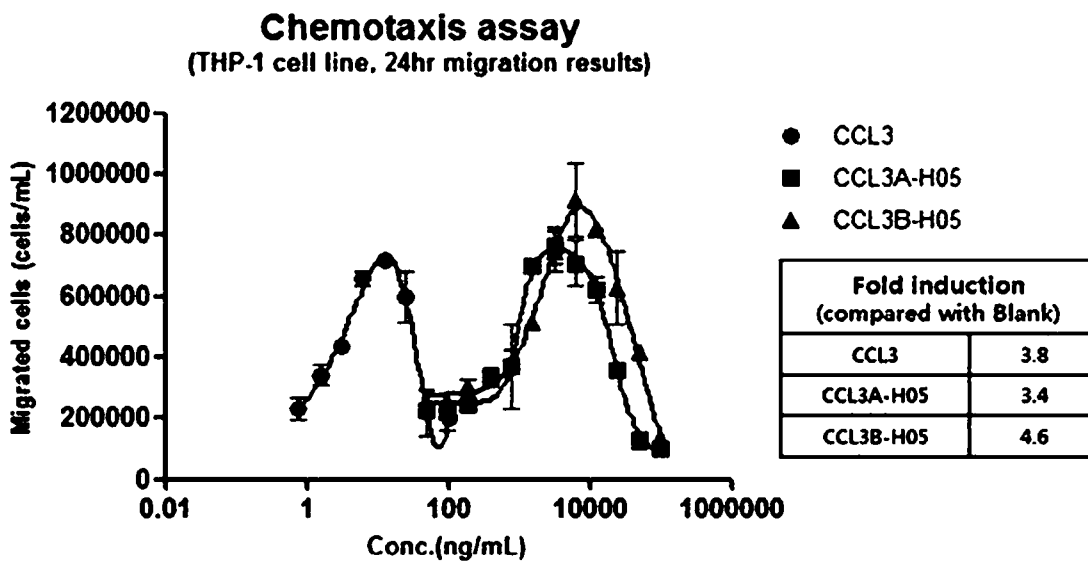
$$(RNT)_nGRGG(EKKK)_m$$
 wherein n and m are respectively 1 to 5.
4. The fusion protein according to claim 3, wherein the linker comprises a sequence as defined in any one of SEQ ID NOs: 5 to 13.
5. The fusion protein according to claim 1, wherein the immunoglobulin Fc region is a hybrid Fc comprising one or more Fc region selected from the group consisting of IgG1, IgG2, IgG3, IgG4 and IgD, a fragment thereof, or a combination thereof.
6. The fusion protein according to claim 5, wherein the hybrid Fc comprises hinge and CH2 of IgD Fc and CH2 and CH3 sequences of IgG4 Fc.
7. The fusion protein according to claim 1, wherein the immunoglobulin Fc region comprises the sequence of SEQ ID NO: 14.
8. A nucleic acid encoding the fusion protein according to any one of claims 1 to 7.
9. A vector comprising the nucleic acid according to claim 8.

10. A cell transformed by the vector according to claim 9.
11. A method of preparing a fusion protein according to any one of claims 1 to 7, comprising the steps of:
 - (a) culturing the cell according to claim 10; and
 - (b) collecting the fusion protein in the cultured cell.
12. A pharmaceutical composition comprising the fusion protein according to any one of claims 1 to 7 and a pharmaceutically acceptable carrier.
13. The pharmaceutical composition according to claim 12, wherein the composition is for treating or preventing cancer.
14. The pharmaceutical composition according to claim 13, wherein the composition is for combining with or assisting radiation treatment.
15. Use of the fusion protein of any one of claims 1-7 for treatment or prevention of cancer.
16. Use of the fusion protein of any one of claims 1-7 in combination with radiation treatment for treatment or prevention of cancer.
17. Use of the fusion protein of any one of claims 1-7 in the manufacture of a medicament for treatment or prevention of cancer.

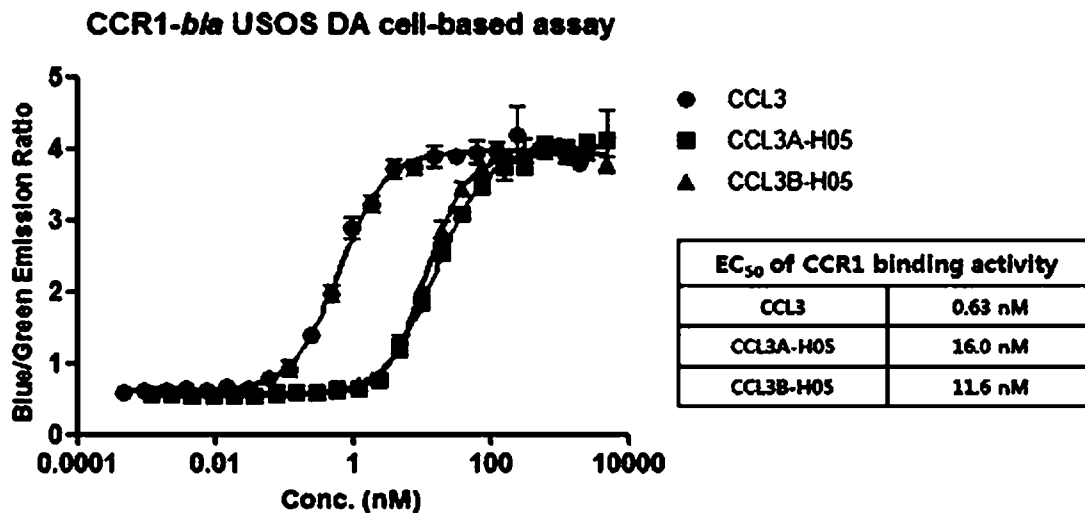
[Fig. 1]



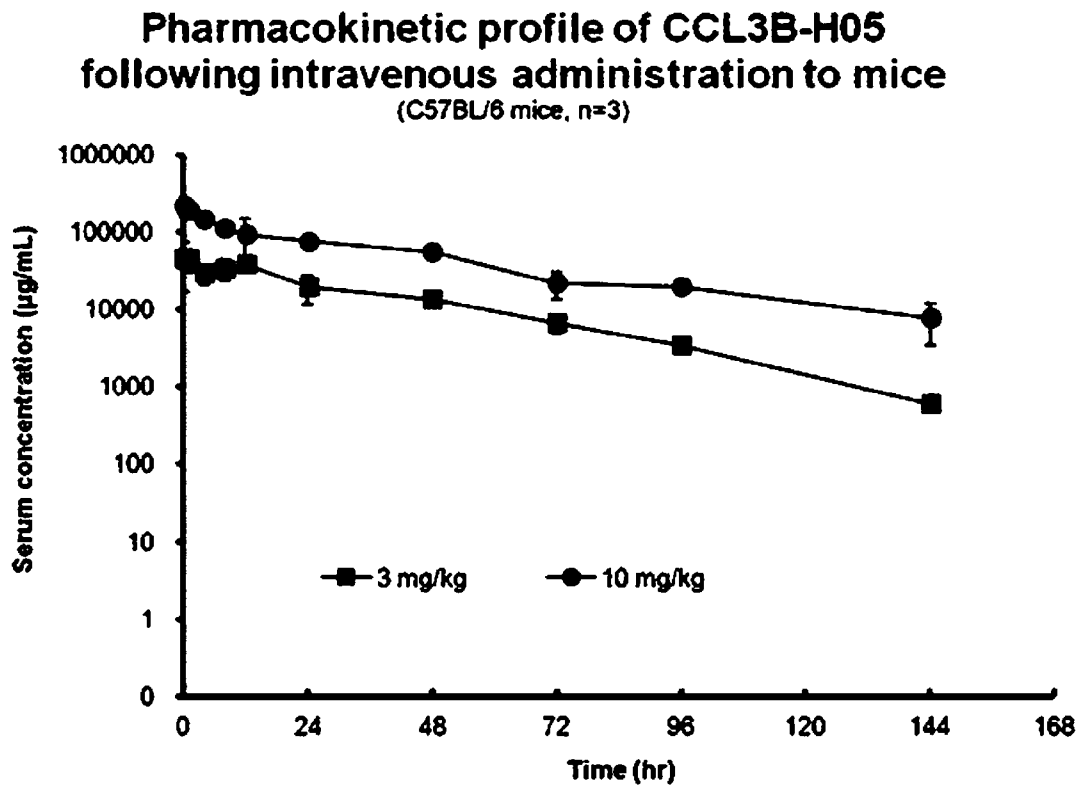
[Fig. 2]



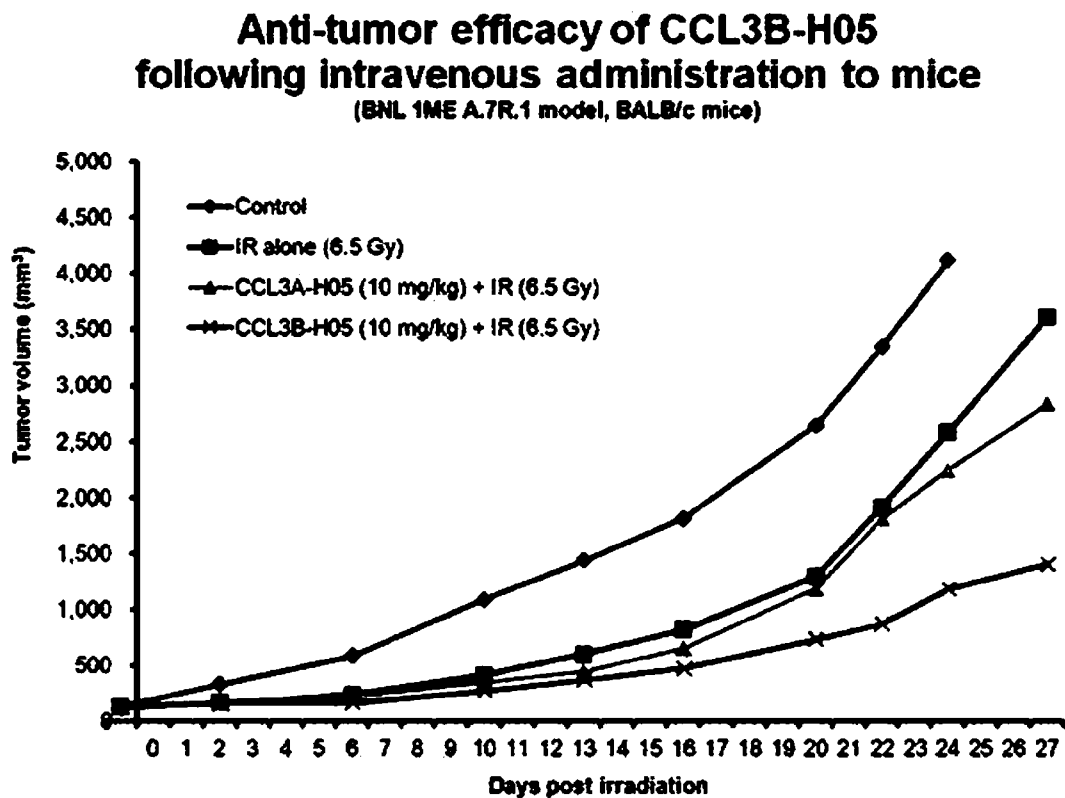
[Fig. 3]



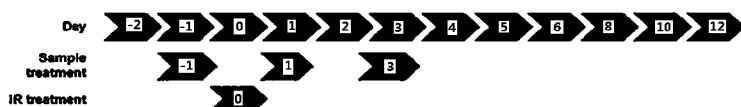
[Fig. 4]



[Fig. 5]



[Fig. 6]



Anti-tumor efficacy of CCL3B-H05 following intravenous administration to mice (BNL 1ME A.7R.1 model, BALB/c mice)

