COMPOSITION FOR PREVENTING OR TREATING AMYOTROPHIC LATERAL SCLEROSIS USING TWO OR MORE ISOFORMS OF HEPATOCYTE GROWTH FACTOR

Technical Field

The present application claims priorities from Korean Patent Application No. 10-2013-0126216 filed with the Korean Intellectual Property Office on 22 October 2013 and Korean Patent Application No. 10-2014-0143377 filed with the Korean Intellectual Property Office on 22 October 2014, the disclosures of which are incorporated herein by reference.

The present invention relates to a composition, containing, as an active ingredient, two or more isoforms of hepatocyte growth factor or a polynucleotide encoding the isoforms, for preventing or treating amyotrophic lateral sclerosis.

Background Art

Amyotrophic lateral sclerosis (ALS), which is a motor neuron disease, was first reported in 1869 by French doctor Jean-Martin Chartcot. ALS was known to normal people since Lou Gehrig, a famous baseball player in the United State who suffered from this disease, in 1939, and from this moment, ALS was called Lou Gehrig`s disease.

The prognosis of ALS is based on clinical features, electric diagnosis tests, and the exclusion of other health states associated with the symptoms. The molecular genetic test, which can be used in clinical tests associated with some genes involved in ALS, plays an important role in genetic type determination and genetic counseling.

ALS may be inherited in an autosomal dominant, autosomal recessive, or X-linked manner. Genetic

counseling and risk assessment depend on the accurate diagnosis of particular genes.

Riluzole has been known as a drug used to delay the progress of ALS. It is known that Riluzole can lower the rate of ALS progress by inhibiting excessive glutamic acid, which is considered to be one of the causes of motor neuron destruction. However, the clinical effects of Riluzole fail to alleviate ALS symptoms, and the results thereof are also not obvious in extending the tracheostomy-free survival of ALS patients receiving no tracheotomy. As described above, the genuine clinical effects of Riluzole, which is helpful to ALS patients, have been reported to be very restricted and obscure (Stewart et al, 2001). Nevertheless, there is no effective preventive or therapeutic agent for ALS, excluding Riluzole having even equivocal clinical effectiveness, and thus the development of drugs exhibiting the effects of preventing or treating ALS is needed.

Meanwhile, expression vectors as a gene delivery system for genetic therapy have been known in the conventional art. The detailed descriptions of pCK vector used in an example of the present invention are disclosed in PCT/KR1999/000855. In addition, PCT/KR2003/000548 discloses a composition, containing pCK-HGFX7 recombinant vector used in the present invention, for treating or preventing ishemic diseases or liver disorders. The entire contents of PCT/KR1999/000855 and PCT/KR2003/000548 are incorporated herein by reference.

Throughout the entire specification, many papers and patent documents are referenced and their citations are represented. The disclosure of the cited papers and patent documents are entirely incorporated by reference into the present specification and the level of the

technical field within which the present invention falls, and the details of the present invention are explained more clearly.

Detailed Description of the Invention

Technical Problem

The present inventors researched and endeavored to develop drugs capable of preventing or treating amyotrophic lateral sclerosis (ALS). As a result, the present inventors established that ALS can be treated by using a composition containing, as an active ingredient, two or more isoforms of hepatocyte growth factor (HGF) or a polynucleotide encoding the isoforms, and thus have completed the present invention.

Therefore, an aspect of the present invention is to provide a pharmaceutical composition for preventing or treating amyotrophic lateral sclerosis.

Another aspect of the present invention is to provide a method for preventing or treating amyotrophic lateral sclerosis.

Other purposes and advantages of the present disclosure will become more obvious with the following detailed description of the invention, claims, and drawings.

Technical Solution

In accordance with an aspect of the present invention, there is provided a pharmaceutical composition for preventing or treating amyotrophic lateral sclerosis, the composition containing, as an active ingredient, two or more isomers of hepatocyte growth factor (HGF) or a polynucleotide encoding the isomers.

The present inventors researched and endeavored to develop drugs capable of preventing or treating

amyotrophic lateral sclerosis. As a result, the present inventors established that ALS could be treated by using a composition containing, as an active ingredient, two or more isoforms of hepatocyte growth factor (HGF) or a polynucleotide encoding the isoforms.

The therapy strategy of the present invention may be largely classified into two types: protein therapy and gene therapy. According to the protein therapeutic agent strategy of the present invention, two or more types of isomeric proteins of HGF are used. Meanwhile, according to the gene therapeutic agent strategy of the present invention, at least one nucleotide sequence encoding two or more types of isomers of HGF is used. A two or more HGF isoforms-encoding polynucleotide sequence may be provided by one polynucleotide or separate polynucleotides. Preferably, the two or more HGF isoforms-encoding polynucleotide sequence is provided by one polynucleotide.

Hereinafter, the present invention will be described in detail.

As used herein, the term “HGF isoform (or isomer of HGF)” refers to an HGF polypeptide having an amino acid sequence that is at least 80% identical to the naturally occurring HGF amino acid sequence in an animal, including all allelic variants. For example, the HGF isoform has a meaning that includes all of a normal form or a wild type of HGF and various variants of HGF (e.g., splicing variants and deleted variants).

In an embodiment of the present invention, the two or more isoforms of HGF include full-length HGF (flHGF) and deleted variant HGF (dHGF). The use of a composition containing both the full-length HGF and the deleted variant HGF can prevent or treat ALS very effectively.

As used herein, the term “flHGF” refers to a

sequence of amino acids 1-728 of the HGF protein from an animal, preferably a mammal, and more preferably a human.

As used herein, the term “dHGF” refers to a deleted variant of the HGF protein produced by alternative splicing of the HGF gene from an animal, preferably a mammal, and more preferably refers to human HGF consisting of 723 amino acids, with the deletion of five amino acids (F, L, P, S, and S) in the first kringle domain of the alpha chain from the full-length HGF sequence.

In an embodiment of the present invention, the full-length HGF of the present invention includes the amino acid sequence of SEQ ID NO: 1, and the deleted variant HGF of the present invention includes the amino acid sequence of SEQ ID NO: 2.

In an embodiment of the present invention, the HGF isoforms of the present invention are encoded by separate nucleotide sequences or a single polynucleotide sequence. Herein, the pharmaceutical composition of the present invention includes two or more polynucleotides when the different types of isoforms of HGF are encoded by separate polynucleotides, and includes at least one polynucleotide including the single polynucleotide when the different types of isoforms of HGF are encoded by the single polynucleotide sequence. The polynucleotide of the present invention may be operatively linked to at least one regulatory sequence (e.g., a promoter or an enhancer) regulating the expression of the HGF isoforms.

When the two or more types of isoforms of HGF are encoded by separate polynucleotides, an expression cassette may be constructed in two manners. According to a first manner, the expression cassette is constructed by linking an expression regulatory sequence to a coding sequence (CDS) of each isoform. According to a second manner, the expression cassette is constructed by using

an internal ribosomal entry site (IRES) and 2A peptides, like “expression regulatory sequence – first isomer CDS – IRES - second isomer CDS - transcription termination sequence”. The IRES allows the gene translation to start at the IRES sequence, thereby expressing two or more genes of interest in the same construct.

When two or more types of isoforms of HGF are encoded by a single polynucleotide, the polynucleotide encoding all the two or more types of isoforms is operatively linked to a single expression regulatory sequence.

In the present invention, the HGF isoforms may be encoded by a hybrid HGF gene that simultaneously expresses two or more different types of isoforms of HGF, e.g., flHGF and dHGF.

According to a preferable embodiment of the present invention, the hybrid HGF gene includes cDNA corresponding exons 1 to 18 of human HGF and intron 4 of the human HGF gene or a fragment thereof, which is inserted between exon 4 and exon 5 of the cDNA.

According to a more preferable embodiment of the present invention, the hybrid HGF gene includes a nucleotide sequence selected from the group consisting of SEQ ID NO: 3 to SEQ ID NO: 10.

The hybrid HGF gene including intron 4 is 7113 bp long and includes the nucleotide sequence of SEQ ID NO: 3. The hybrid HGF gene may selectively include a fragment of intron 4 between exon 4 and exon 5 of HGF cDNA.

According to a preferable embodiment of the present invention, the sequence additionally inserted between exon 4 and exon 5 includes: intron 4 of the human HGF gene, nucleotides 392-2247, nucleotides 392-727, nucleotides 2229-5471, nucleotides 5117-5471, nucleotides 3167-5471, nucleotides 4167-5471, or a

combination thereof, of the nucleotide sequence of SEQ ID NO: 3.

More preferably, the sequence additionally inserted between exon 4 and exon 5 of the therapeutic nucleotide sequence used in the present invention is (i) nucleotides 392-2247 and nucleotides 2229-5471 of SEQ ID NO: 3; (ii) nucleotides 392-2247 and nucleotides 5117-5471 of SEQ ID NO: 3; (iii) nucleotides 392-2247 and nucleotides 3167-5471 of SEQ ID NO: 3; (iv) nucleotides 392-2247 and nucleotides 4167-5471 of SEQ ID NO: 3; (v) nucleotides 392-727 and nucleotides 2229-5471 of SEQ ID NO: 3; (vi) nucleotides 392-727 and nucleotides 5117-5471 of SEQ ID NO: 3; (vii) nucleotides 392-727 and nucleotides 3167-5471 of SEQ ID NO: 3; or (viii) nucleotides 392-727 and nucleotides 4167-5471 of SEQ ID NO: 3.

The therapeutic nucleotide sequence of the present invention according to the sequence additionally inserted between exon 4 and exon 5 is summarized as below: (i) (exon 1 to exon 4)-(nucleotides 392-2247 - nucleotides 2297-5471 of SEQ ID NO: 3)-(exon 5 to exon 18); (ii) (exon 1 to exon 4)-(nucleotides 392-2247 - nucleotides 5117-5471 of SEQ ID NO: 3)-(exon 5 to exon 18); (iii) (exon 1 to exon 4)-(nucleotides 392-2247 - nucleotides 392-5471 of SEQ ID NO: 3)-(exon 5 to exon 18); (iv) (exon 1 to exon 4)-(nucleotides 392-2247 - nucleotides 4167-5471 of SEQ ID NO: 3)-(exon 5 to exon 18); (v) (exon 1 to exon 4)-(nucleotides 392-727 - nucleotides 2229-5471 of SEQ ID NO: 3)-(exon 5 to exon 18); (vi) (exon 1 to exon 4)-(nucleotides 392-727 - nucleotides 5117-5471 of SEQ ID NO: 3)-(exon 5 to exon 18); (vii) (exon 1 to exon 4)-(nucleotides 392-727 - nucleotides 3167-5471 of SEQ ID NO: 3)-(exon 5 to exon 18); and (viii) (exon 1 to exon 4)-(nucleotides 392-727 - nucleotides 4167-5471 of SEQ ID NO: 3)-(exon 5 to exon

18).

Herein, the hybrid HGF gene including the fragment of intron 4 is named “HGF-X”, and the HGF-X includes HGF-X2, HGF-X3, HGF-X4, HGF-X5, HGF-X6, HGF-X7, and HGF-X8, which have nucleotide sequences of SEQ ID NOs: 4 to 10. In the present invention, HGF-X7 is preferably used. The “HGF isoform”, “HGF-X”, and “HGF-X7” in the present invention have been reported in PCT/KR2003/000548, the disclosure of which is incorporated herein by reference.

Amino acid or nucleotide sequences of the HGF isoforms, which may be used in the present invention, are construed to include amino acid or nucleotide sequences having substantial identity to the sequences of wild type human HGF isoforms. The term “substantial identity” means that, when the amino acid or nucleotide sequence of the wild type human HGF isoform and another nucleotide sequence are aligned to correspond to each other as much as possible and the aligned sequences are analyzed using an algorithm that is normally used in the art, the amino acid or nucleotide sequence of the wild type human HGF isoform shows at least 80% identity, preferably at least 90% identity, and most preferably at least 95% identity. Methods of alignment for the sequence comparison are well known in the art. Various methods and algorithms for alignment are disclosed in Smith and Waterman, Adv. Appl. Math. 2:482 (1981); Needleman and Wunsch, J. Mol. Bio. 48:443 (1970); Pearson and Lipman, Methods in Mol. Biol. 24:307-31 (1988); Higgins and Sharp, Gene 73:237-44 (1988); Higgins and Sharp, CABIOS 5:151-3 (1989); Corpet et al., Nuc. Acids Res. 16:10881-90 (1988); Huang et al., Comp. Appl. BioSci. 8:155-65 (1992); and Pearson et al., Meth. Mol. Biol. 24:307-31 (1994). The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., J. Mol. Biol. 215:403-10 (1990)) is available via the National

Center for Biological Information (NCBI) and, on the Internet, may be used in connection with the sequence analysis programs, such as blastp, blasm, blastx, tblastn, and tblastx. BLSAT can be accessed through http://www.ncbi.nlm.nih.gov/BLAST/. The sequence identity comparision method using such a program can be confirmed in http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html.

As used herein, the term “prevention” refers to all the actions of suppressing amyotrophic lateral sclerosis or delaying the progress of amyotrophic lateral sclerosis through administration of the composition of the present invention.

The term “treatment” used herein refers to (a) the suppression of the progress of amyotrophic lateral sclerosis, (b) the relief of amyotrophic lateral sclerosis, or (c) the removal of amyotrophic lateral sclerosis.

The composition of the present invention can prevent or treat amyotrophic lateral sclerosis through the neurite outgrowth and growth as well as the growth and anti-apoptosis of motor neurons.

The composition of the present invention may be applied *in vivo* through various delivery methods that are conventionally known in the gene therapy field.

In an embodiment of the present invention, the polynucleotide of the present invention is naked DNA or is contained in a gene delivery system. Examples of the gene delivery system include a plasmid, a vector, and a viral vector.

(i) Plasmid (vector)

A plasmid (vector) may be used as a delivery system that delivers the polynucleotide of the present invention. The polynucleotide included in the vector

preferably exists in an appropriate expression cassette. Preferably, the polynucleotide is operatively linked to a promoter in the expression cassette.

As used herein, the term “operatively linked” refers to a functional linkage between a nucleic acid expression regulatory sequence (e.g., a promoter, signal sequence, or an array at the binding site of a transcription regulation factor) and another nucleic acid sequence, and through the linkage, the regulatory sequence regulates the transcription and/or translation of another nucleic acid sequence.

In the present invention, the promoter binding to the polynucleotide sequence is one that can regulate the transcription of the nucleotide sequence by operating in animal cells, preferably mammalian cells, and more preferably human cells, and includes, for example, promoters derived from mammalian viruses and promoters derived from mammalian cell genomes. Examples thereof may include cytomegalovirus (CMV) promoter, adenovirus late promoter, vaccinia virus 7.5K promoter, SV40 promoter, HSV tk promoter, RSV promoter, EF1 alpha promoter, metallothionein promoter, beta-actin promoter, human IL-2 gene promoter, human IFN gene promoter, human IL-4 gene promoter, human lymphotoxin gene promoter, and human GM-CSF gene promoter, but are not limited thereto. Still more preferably, the promoter used in the present invention is a promoter derived from the human CMV (hCMV) immediately early (IE) gene, or an EF1 alpha promoter, and most preferably, a 5’-untranslated region (UTR) including promoter/enhancer and the entire sequence of exon 1 immediately to ATG initiation codon of exon 2, of the hCMV IE gene.

The expression cassette used in the present invention may include a polyadenylation sequence, for example, a bovine growth hormone terminator (Gimmi, E.

R., et al., Nucleic Acids Res. 17:6983-6998 (1989)), SV40-derived polyadenylation sequence (Schek, N, et al., Mol. Cell Biol. 12:5386-5393 (1992)), HIV-1 polyA(Klasens, B. I. F., et al., Nucleic Acids Res. 26:1870-1876 (1998)), β-globin polyA(Gil, A., et al, Cell 49:399-406 (1987)), HSV TK polyA (Cole, C. N. and T. P. Stacy, Mol. Cell. Biol. 5:2104-2113 (1985)) or polyoma virus poly A (Batt, D. B and G. G. Carmichael, Mol. Cell. Biol. 15:4783-4790 (1995)), but are not limited thereto.

According to a preferable embodiment of the present invention, pCK, pCP, pVAX1, or pCY vector may be used as a delivery system of the polynucleotide, and more preferably, pCK vector may be used. The pCK vector is disclosed in detail in WO 2000/040737, the disclosure of which is incorporated herein by reference.

(ii) Retrovirus

Retrovirus can introduce a gene thereof into the genome of a host to deliver a lot of exotic genetic materials, and has a wide spectrum of infectible cells, so most retroviruses are used as a gene delivery vector.

In order to construct the retroviral vector, the polynucleotide sequence of the present invention is inserted into the retroviral genome but not the retroviral sequence, thereby producing replication-defective viruses. For virion production, a packaging cell line containing gag, pol, and env genes but having no long terminal repeat (LTR) sequence and ψ sequence is constructed (Mann et al., Cell, 33:153-159 (1983)). When the recombinant plasmid containing the polynucleotide sequence of the present invention, the LTR sequence, and the ψ sequence is introduced into the cell line, the ψ sequence allows the production of RNA transcripts of the recombinant plasmid, and these transcripts are packaged

with viruses, which are discharged to the media (Nicolas and Rubinstein "Retroviral vectors," In: Vectors: A survey of molecular cloning vectors and their uses, Rodriguez and Denhardt (eds.), Stoneham: Butterworth, 494-513 (1988)). The media containing the recombinant retroviruses are collected and concentrated, and then used as a gene delivery system.

The gene delivery using second-generation retroviral vectors has been published. Kasahara et al., manufactured a moloney murine leukemia virus variant, and produced a chimeric protein having new binding characteristics by inserting the erythropoietin (EPO) sequence into the envelope site thereof (Science, 266:1373-1376 (1994)). The polynucleotide sequence of the present invention may also be introduced into the retrovirus according to the construction strategy of the second-generation retrovirus vector.

(iii) Adenovirus

Adenovirus has usually been employed as a gene delivery vector due to the mid-sized genome, ease of engineering, high titer, wide range of target cells, and high infectivity. Both ends of the genome contain 100-200 bp inverted terminal repeats (ITRs), which are cis-elements necessary for DNA replication and packaging. E1 region (E1A and E1B) of the genome encodes proteins responsible for the regulation of transcription of the viral genome and the transcription of host cell genes. E2 region (E2A and E2B) encodes the proteins involved in viral DNA replication.

Out of the adenoviral vectors developed so far, the replication-defective adenovirus having the deleted E1 region is usually used. Meanwhile, the deleted E3 region in normal adenoviral vectors may provide an insertion site for exotic genes (Thimmappaya, B. et al., Cell,

31:543-551 (1982); and Riordan, J. R. et al., Science, 245:1066-1073 (1989)). Therefore, the polynucleotide sequence of the present invention is preferably inserted into either the deleted E1 region (E1A region and/or E1B region) or the deleted E3 region. In addition, the polynucleotide sequence may also be inserted into the deleted E4 region. Herein, the term “deletion” used with reference to viral genome sequences encompasses the complete deletion of the corresponding sequence as well as the partial deletion thereof. In addition, the adenovirus can package approximately 105% of the wild-type genome, providing capacity for about 2 extra kb of DNA (Ghosh-Choudhury et al., *EMBO J.,* 6:1733-1739 (1987)). Therefore, the foregoing exotic sequences inserted into the adenovirus may be further coupled with the adenoviral genome.

Adenovirus may be of any of 42 different serotypes and subgroups A-F. Of these, adenovirus type 5 pertaining to subgroup C is the most preferable starting material for obtaining the adenoviral vector of the present invention. Biochemical and genetic information about adenovirus type 5 has been well known. The exotic genes delivered by the adenovirus are replicated in the same manner as in the episome, and thus have low genotoxicity to host cells. Therefore, the gene therapy using the adenoviral gene delivery system is determined to be safe.

(iv) AAV vector

Adeno-associated viruses (AAV) are capable of infecting non-divided cells and have the ability to infect various types of cells, and thus are suitable as a gene delivery system of this invention. Detailed descriptions for the use and preparation of the AAV vector are disclosed in U.S. Pat. Nos. 5,139,941 and

4,797,368.

Research results for AAV as a gene delivery system are disclosed in LaFace et al, Viology, 162:483486 (1988), Zhou et al., Exp. Hematol. (NY), 21:928-933 (1993), Walsh et al, J. Clin. Invest., 94:1440-1448 (1994), and Flotte et al., Gene Therapy, 2:29-37 (1995). Recently, the AAV vector has been approved for Phase I human trials for the treatment of cystic fibrosis.

Typically, the AAV virus is manufactured by co-transfecting a plasmid containing a target gene sequence flanked by two AAV terminal repeats (McLaughlin et al., J. Virol., 62:1963-1973 (1988); and Samulski et al., J. Virol., 63:3822-3828 (1989)) and an expression plasmid containing a wild type AAV coding sequence without terminal repeats (McCarty et al., J. Virol., 65:2936-2945 (1991)).

(v) Other Viral Vectors

Other viral vectors may be used to deliver the polynucleotide sequence of the present invention into the biology body. Vectors derived from viruses, such as vaccinia virus (Puhlmann M. et al., Human Gene Therapy 10:649-657 (1999); Ridgeway, "Mammalian expression vectors," In: Vectors: A survey of molecular cloning vectors and their uses. Rodriguez and Denhardt, eds. Stoneham: Butterworth, 467-492 (1988); Baichwal and Sugden, "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press, 117-148 (1986) and Coupar et al., Gene, 68:1-10 (1988)), lentivirus (Wang G. et al., J. Clin. Invest. 104(11):R55-62 (1999)), or herpes simplex virus (Chamber R., et al., Proc. Natl. Acad. Sci USA 92:1411-1415 (1995)) may also be used as a delivery system capable of delivering the polynucleotide

into cells.

(vi) Liposomes

Liposomes are formed spontaneously by phospholipids suspended in the aqueous medium. Liposome-mediated exotic DNA molecule delivery has been very successful as described in Nicolau and Sene, Biochim. Biophys. Acta, 721:185-190 (1982) and Nicolau et al., Methods Enzymol., 149:157-176 (1987). Liposomes entrapping the polynucleotide sequence of the present invention delivery the polynucleotide sequence into cells by interacting with cells through mechanisms, such as endocytosis, adsorption onto cell surfaces, and fusion with plasma cellular membranes.

In cases where the polynucleotide sequence of the present invention is introduced in a naked recombinant DNA molecule or a plasmid (vector), the polynucleotide sequence may be introduced into cells by micro-injection (Capecchi, M.R., Cell, 22:479 (1980); and Harland & Weintraub, J. Cell Biol. 101:1094-1099 (1985)), phosphate calcium precipitation (Graham, F.L. et al., Virology, 52:456 (1973); and Chen & Okayama, Mol. Cell. Biol. 7:2745-2752 (1987)), electroporation (Neumann, E. et al., EMBO J., 1:841 (1982); and Tur-Kaspa et al., Mol. Cell Biol., 6:716-718 (1986)), liposome-mediated transfection (Wong, T.K. et al., Gene, 10:87 (1980); Nicolau & Sene, Biochim. Biophys. Acta, 721:185-190 (1982); and Nicolau et al., Methods Enzymol., 149:157-176 (1987)), DEAE-dextran treatment (Gopal, Mol. Cell Biol., 5:1188-1190 (1985)) and gene bambardment (Yang et al., Proc. Natl. Acad. Sci., 87:9568-9572 (1990)).

When the polynucleotide sequence of the present invention is constructed based on the viral vector, the polynucleotide sequence may be delivered into cells by various viral infection methods known in the art. The

infection of host cells using viral vectors are described in the above-mentioned cited documents.

In a preferable embodiment of the present invention, the gene delivery system of the present invention is a vector.

In an embodiment of the present invention, the vector of the present invention is a plasmid, and most preferably, the pCK vector may be used. An example of the recombinant vector including a single polynucleotide expressing two or more isomers of HGF using the pCK vector may be pCK-HGFX7, the contents of which are described in detail in PCT/KR1999/000855 and PCT/KR2003/000548, as described above.

The composition of the present invention may contain a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier contained in the composition of the present invention is conventionally used for the formulation, and examples thereof may include, but are not limited to, lactose, dextrose, sucrose, sorbitol, mannitol, starch, acacia gum, calcium phosphate, alginate, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, methyl cellulose, methyl hydroxybenzoate, propyl hydroxybenzoate, talc, magnesium stearate, and mineral oil. The pharmaceutical composition of the present invention may further contain a lubricant, a wetting agent, a sweetening agent, a flavoring agent, an emulsifier, a suspending agent, a preservative, and the like, in addition to the above ingredient. Suitable pharmaceutically acceptable carriers and agents are described in detail in *Remington's Pharmaceutical Sciences* (19th ed., 1995).

Preferably, the pharmaceutical composition of this invention may be administered parenterally, and for example, intravenous administration, intraperitoneal

administration, subcutaneous administration, intradermal administration, intraspinal administration, intrathecal administration, intraventricular administration, parenchymal administration, intracranial administration, intramuscular administration, or local administration may be employed. Most preferably, the pharmaceutical composition of this invention may be administered intramuscularly, spinally, intrathecally, intraventricularly, parenchymally, or intracranially.

The pharmaceutical composition of the present invention may be formulated and administered as an injection. The appropriate dose of the pharmaceutical composition of the present invention varies depending on factors, such as the formulating method, manner of administration, patient's age, body weight, gender, and severity of disease, time of administration, route of administration, excretion rate, and response sensitivity, and the ordinarily skilled practitioner can easily judge and prescribe the dose effective for desired treatment or prevention.

According to a preferable embodiment of the present invention, the isoforms of HGF of the present invention are administered at a dose of 1 μg to 2,500 mg for each, and the polynucleotide encoding the isoforms is administered at a dose of 1 μg to 2,500 mg. When the isoforms of HGF or the polynucleotide encoding the isoforms is repeatedly administered once or more, the dose may be equal or different for each administration.

The pharmaceutical composition of the present invention is formulated using a pharmaceutically acceptable carrier and/or excipient, according to the method that is easily conducted by person having ordinary skills in the art to which the present invention pertains, and the pharmaceutical composition may be prepared into a unit dosage form or may be

inserted into a multidose container. Here, the dosage form may be a solution in an oily or aqueous medium, a suspension, an emulsion, an extract, a powder, granules, a tablet, or a capsule, and may further contain a dispersant or a stabilizer.

In accordance with another aspect of the present invention, there is provided a method for preventing or treating amyotrophic lateral sclerosis, the method including administering, to a mammal, a composition containing, as an active ingredient, two or more isomers of hepatocyte growth factor (HGF) or a polynucleotide encoding the isomers.

In an embodiment of the present invention, the two or more HGF isoforms of the present invention include full-length HGF (flHGF) and deleted variant HGF (dHGF).

In an embodiment of the present invention, the full-length HGF of the present invention includes the amino acid sequence of SEQ ID NO: 1, and the deleted variant HGF of the present invention includes the amino acid sequence of SEQ ID NO: 2.

Since the method for preventing or treating amyotrophic lateral sclerosis of the present invention includes the step of administering the pharmaceutical composition for preventing or treating amyotrophic lateral sclerosis, which is an aspect of the present invention, the overlapping descriptions therebetween are omitted to avoid excessive complication of the specification due to repetitive descriptions thereof.

Advantageous Effects

Features and advantages of the present invention are summarized as follows:

(a) The present invention provides a pharmaceutical composition for preventing or treating amyotrophic lateral sclerosis.

(b) The present invention provides a method for preventing or treating amyotrophic lateral sclerosis.

(c) The composition or method of the present invention may be used to prevent or treat amyotrophic lateral sclerosis through the neurite outgrowth and growth in embryonic neural cells as well as the growth and anti-apoptosis of motor neurons.

Brief Description of the Drawings

FIG. 1 depicts an effect of pCK-HGFX7 on the neurite outgrowth of ENC cells according to an embodiment of the present invention.

FIG. 2 depicts an effect of pCK-HGFX7 on the growth of ENC cells according to an embodiment of the present invention.

FIG. 3 depicts an effect of pCK-HGFX7 on cell growth in NSC-34 cells according to an embodiment of the present invention.

FIG. 4 depicts an effect of pCK-HGFX7 on apoptosis in NSC-34 cells according to an embodiment of the present invention.

FIG. 5 depicts an effect of pCK-HGFX7 on the survival of NSC-34 cells under oxidative stress culture conditions according to an embodiment of the present invention.

FIG. 6 depicts an effect of pCK-HGFX7 on apoptosis of NSC-34 cells under oxidative stress culture conditions according to an embodiment of the present invention.

FIG. 7 depicts an effect of pCK-HGFX7 on cell growth in the G93A mutant hSOD1-delivered cells according to an embodiment of the present invention.

FIG. 8 depicts an effect of pCK-HGFX7 on grip strength in ALS mice according to an embodiment of the present invention.

Mode for Carrying Out the Invention

Hereinafter, the present invention will be described in detail with reference to examples. These examples are only for illustrating the present invention more specifically, and it will be apparent to those skilled in the art that the scope of the present invention is not limited by these examples.

Examples

Example 1: Verification on effect of pCK-HGFX7 on maturation of embryonic neuronal cell (ENC)

Only the cerebral cortex portion was taken from the mouse embryo to be made into single cells, and then 10 μM Ara-C 10 was added to the culture medium to culture only neuronal cells. In order to verify the effect of pCK-HGFX7 on the maturation of ENC, 2×10^4 cells were seeded, and the next day, the cells were treated with 1.25 ng/㎖ of the protein obtained from 293F cells (Life technologies, USA) transfected with pCK-HGFX7, thereby verifying the degree of neurite outgrowth shown in the cell maturation. The degree of neurite outgrowth was confirmed through immunocytochemistry on the expression of TUJ-1, which is a tubulin protein expressed specifically to neuronal cells.

The results confirmed that, as shown in FIG. 1, the neurite length was significantly increased in the pCK-HGFX7 treatment group rather than the pCK treatment group as a control.

Example 2: Verification on effect of pCK-HGFX7 on cell growth after ENC maturation

The effect of pCK-HGFX7 on cell growth after ENC maturation was verified. To this end, 5×10^4 ENCs were

seeded, followed by maturation for 6 days. After 6 days, the cells were treated with 1.25 ng/㎖ of the protein obtained from 293F cells transfected with pCK-HGFX7, thereby verifying the effect of pCK-HGFX7 on cell growth. After 3 days of the treatment with pCK-HGFX7, an MTT assay was carried out to measure the cell growth.

The results confirmed that, as shown in FIG. 2, the cell growth was significantly increased by about 40% in the pCK-HGFX7 treatment group rather than the pCK treatment group as a control.

Example 3: Verification on effect of pCK-HGFX7 on cell growth and apoptosis in mouse motor neuronal cells (NSC-34)

3-1. Cell line and cell culture

NSC-34 cells (Cellution Biosystem, Vancouver, CA) used in the present test are mouse-derived motor neuronal cells. NSC-34 cells correspond to a cell line in which motor neuronal cells derived from the spinal nerve of the embryonic mouse are mixed with neuroblastoma cells, and are widely used in studies associated with the motor nerve. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% bovine fetal serum and an antibiotic material (Gibco BRL, USA) under the conditions of 37℃and 5% CO_2. The medium, reagent, and serum for cell culture were purchased from Gibco and Sigma aldrich.

3-2. Production and quantification of supernatant expressing HGF protein

DNA transfection was used to produce the supernatant expressing HGF protein. The transfection was carried out using the FuGene HD transfection system (Promega, USA) according to the manufacturer's protocol.

293T cells were seeded at 1×10^6 cells, and the next day, the cells were transfected with 3 ㎍ of pCK, pCK-HGF728 (pCK-cHGF in PCT/KR03/000548), pCK-HGF723 (pCK-dHGF in PCT/KR03/000548), and pCK-HGFX7 DNA. After culture for 48 h, respective supernatants were all harvested, and then filtered through a 0.22-㎛ filter. The expression level of the HGF protein contained in each supernatant was measured using human HGF immunoassay. Each supernatant was again diluted to 1 ㎍/㎖ for the use of tests. Recombinant human HGF protein used in the human HGF immunoassay was purchased from R&D (R&D Systems, Inc., MSP, USA) for use.

3-3. Effect of human pCK-HGFX7 on cell growth in NSC-34 cells

In order to verify the effect of pCK-HGFX7 on the growth of motor neuronal cells, NSC-34 cells were treated with pCK-HGFX7, and then the degree of cell proliferation was evaluated. The cells were cultured in a culture medium supplemented with 10% bovine fetal serum, and then, for the use for tests, the culture was suspended using Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 1% bovine fetal serum. The cells were seeded in a 6-well plate such that 3×10^4 of the cells were contained in 2 ㎖ of a medium containing 1% serum. After 2 h of the seeding, respective supernatants obtained from 293T cells transfected with pCK-HGF728, pCK-HGF723, and pCK-HGFX7 were added to a 6-well plate at 100 uL per well such that the concentration of the HGF protein is 50 ng/㎖. The supernatant obtained by transfecting 293T cells with pCK vector was used as a control. After 48 h of culturing, the media in the 6-well plate were exchanged. After 2 ㎖ of a medium containing 1% bovine fetal serum was added to each well, each of the supernatants obtained from

293T cells transfected with pCK-HGF728, pCK-HGF723, and pCK-HGFX7 was added such that the concentration of the HGF protein is 50 ng/㎖, followed by culturing for 48 h. The supernatant obtained by transfecting 293T cells with pCK vector was used as a control. The cultured cells were collected and counted. The pCK vector was used for a control.

As a result of culturing for 5 days after cell seeding, when the groups treated with respective supernatants obtained from 293T cells transfected with pCK, pCK-HGF728, pCK-HGF723, and pCK-HGFX7 were compared with the pCK treatment group, the cell proliferation was induced by about 20% in the pCK-HGF728 or pCK-HGF723 treatment group and the cell growth was increased by about 48% in the pCK-HGFX7 treatment group. Through these results, it could be verified that pCK-HGFX7 can significantly increase the cell growth of motor neuronal cells compared with pCK-HGF728 or pCK-HGF723.

3-4. Effect of pCK-HGFX7 on apoptosis in NSC-34 cells

NSC-34 cells were suspended at 3×10^4 in Dulbecco's modified Eagle's medium supplemented with 1% bovine fetal serum, and then seeded in a 6-well plate. After the seeding of cells, the cells were stabilized for 2 h, and then each of the supernatants obtained from 293T cells transfected with pCK-HGF728, pCK-HGF723, and pCK-HGFX7 was added such that the concentration of HGF protein was 50 ng/㎖. The supernatant obtained by transfecting 293T cells with pCK vector was used as a control. The cells were cultured for 5 days while the medium and each of the supernatants were exchanged at intervals of 2-3 days.

RNA was extracted from the cells, which were cultured for 5 days, using Trizol reagent (Life

technologies, USA), and the extracted RNA was used to synthesize cDNA using First Strand cDNA Kit (Roche, USA). Real-time polymerase chain reaction (PCR) was conducted using the synthesized cDNA as a template and the nucleotides of SEQ ID NOs: 11 and 12 for Bax gene or the nucleotides of SEQ ID NOs: 13 and 14 for Bcl-12 gene as primers. Real-time PCR was performed by mixing 1 ㎕ of the template cDNA, 1 ㎕ of 10 pmol/㎕ primers each, 12.5 ㎕ of SYBR green PCR master mix (Life technologies, USA), and 9.5 ㎕ of sterilized tertiary distilled water to prepare a total of 25 ㎕ of a mixture liquid and then conducting a reaction under conditions of 2 min at 50℃ and 10 min at 95℃, and then 40 cycles of 15 s at 95℃ and 1 min at 60℃. Here, in order to correct each reaction value, real-time PCR was performed using, as primers, the nucleotides of SEQ ID NOs: 15 and 16 for GAPDH as a housekeeping gene. As test results, the expression of the Bax gene associated with apoptosis was reduced in each of HGF supernatant treatment groups rather than the pCK treatment group, and especially, the expression of Bcl-2 associated with anti-apoptosis was increased by 1.3-fold in the pCK-HGFX7 treatment group rather than the pCK treatment group (see FIG. 4a). Through this test, it was confirmed that, in the medium containing 1% bovine fetal serum, pCK-HGFX7 inhibited apoptosis by about 40%, compared with the pCK treatment group as a control (see FIG. 4b).

Example 4: Verification on effect of pCK-HGFX7 on survival of NSC-34 cells under oxidative stress culture conditions

4-1. Selection of concentration of hydrogen peroxide solution inducing NSC-34 cell apoptosis by oxidative stress

Prior to verification of the effect of pCK-HGFX7 on

NSC-34 cell apoptosis induced by hydrogen peroxide solution, the cell seeding concentration that is suitable to validate NSC-34 cell apoptosis, and the concentration of hydrogen peroxide solution for inducing apoptosis were selected.

The cells cultured in a culture medium supplemented with 10% bovine fetal serum were collected, and then suspended in Dulbecco's modified Eagle's medium supplemented with 1% bovine fetal serum, followed by cell counting. The counted cells were seeded in a 96-well plate at a cell concentration of 1×10^4, and then treated the next day with 10, 20, 30, 50, and 100 ㎛ hydrogen peroxide solutions. The phosphate buffered saline was added to a well that was not treated with hydrogen peroxide solution, and the well was used as a control. After 24 h, the degree of apoptosis was measured using the XTT test method (Roche, USA). It was verified that the test groups treated with different concentrations of hydrogen peroxide solution induced 0, 10, 30, 70, and 85% apoptosis compared with the control. Based on these results, the suitable concentration of hydrogen peroxide solution for inducing NSC-34 cell apoptosis was selected to 30 ㎛.

Additionally, in order to carry out the apoptosis test in a 6-well plate, the cells were seeded in the 6-well plate such that the cells were contained at 1.5×10^5, 3×10^5, and 1×10^6 in 2 ㎖ of media containing 1% bovine fetal serum. The next day, NSC-34 cells were treated with the 30 ㎛ hydrogen peroxide solution, and the cell count was carried out on day 1, 4, and 7. As a result of confirming the cell count on day 7, the cells in the well in which 1.5×10^5 cells were seeded were all dead, and thus cannot be selected for the test, and the apoptosis was not induced in the well in which 1×10^6 cells were seeded. Based on these test results, the cell

count and the concentration of hydrogen peroxide solution in the test for apoptosis induction were selected to 3×10^5 and 30 μM, respectively.

4-2. Verification on effect of pCK-HGFX7 on survival of NSC-34 cells under oxidative stress culture conditions

NSC-34 cells were cultured in a culture medium supplemented with 10% bovine fetal serum, and, for the use for the apoptosis inhibition test, the culture was suspended using Dulbecco's modified Eagle's medium supplemented with 1% bovine fetal serum. The cells were seeded in a 6-well plate such that 3×10^5 of the cells were contained in 2 ml of a medium containing 1% serum. The next day, respective wells were treated with 30 μM hydrogen peroxide solution selected from the prior test, and then treated with respective supernatants obtained from 293T cells transfected with pCK-HGF728, pCK-HGF723, and pCK-HGFX7 such that the concentration of the HGF protein was 50 ng/ml. A culture medium obtained by transfecting cells with pCK vector was used as a control. While the cells were cultured for 7 days, the degree of apoptosis was observed. After 7 days of cell seeding, the cells were collected and counted. As a result of cell counting, it was verified that, only about 60-70% of cells survived as compared with the originally seeded cells in the test groups treated with pCK, pCK-HGF728, and pCK-HGF723, whereas NSC-34 cells treated with pCK-HGFX7 showed about 92% survival, indicating the excellent apoptosis inhibition compared with the groups treated with the other test materials. These results confirmed that the HGFX7 protein effectively inhibited motor neuron apoptosis induced by oxidative stress due to the hydrogen peroxide solution (see FIG. 5).

4-3. Verification on effect of pCK-HGFX7 on NSC-34 cell apoptosis under oxidative stress culture conditions

NSC-34 cells were suspended at 3×10^5 in Dulbecco's modified Eagle's medium supplemented with 1% bovine fetal serum, and then seeded in a 6-well plate. The next day, respective wells were treated with 30 μM hydrogen peroxide solution, and then treated with respective supernatants obtained from 293T cells that were transfected with pCK-HGF728, pCK-HGF723, and pCK-HGFX7 such that the concentration of the HGF protein was 50 ng/㎖, followed by culturing for 7 days. The supernatant obtained by transfecting cells with pCK vector was used as a control.

RNA was extracted from the cells, which were cultured for 7 days, using the Trizol reagent, and the extracted RNA was used to synthesize cDNA using the First Strand cDNA Kit. Real-time polymerase chain reaction (PCR) was conducted using the synthesized cDNA as a template and the nucleotides of SEQ ID NOs: 11 and 12 for Bax gene or the nucleotides of SEQ ID NOs: 13 and 14 for Bcl-12 gene as primers. Real-time PCR was performed by mixing 1 ㎕ of the template cDNA, 1 ㎕ of 10 pmol/㎕ primers each, 12.5 ㎕ of SYBR green PCR master mix (Life technologies, USA), and 9.5 ㎕ of sterilized tertiary distilled water to prepare a total of 25 ㎕ of a mixture liquid and then conducting a reaction under conditions of for 2 min at 50℃and 10 min at 95℃ and then 40 cycles of 15 s at 95℃and 1 min at 60℃ Here, in order to correct each reaction value, real-time PCR was performed using, as primers, the nucleotides of SEQ ID NOs: 15 and 16 for GAPDH as a housekeeping gene. The test results confirmed that the expression of the Bax gene associated with apoptosis induction was reduced and the expression of Bcl-2 associated with anti-apoptosis was increased in each of HGF supernatant treatment

groups rather than the pCK treatment group. Especially, the expression of Bax gene was reduced by about 70% (see FIG. 6a), and the Bax/Bcl-2 ratio was decreased by about 75%, indicating an excellent apoptotic effect (see FIG. 6b), in the pCK-HGFX7 treatment group rather than the pCK treatment group.

Example 5: Verification on effect of pCK-HGFX7 on cell growth in G93A mutant hSOD1-delivered cells

The *in vitro* assay using the G93A mutant form of superoxide dismutase 1 (SOD1), which is one of the ALS causes, has been developed by many researchers. Especially, it has been reported that the delivery of the G93A mutant form of hSOD1 into NSC-34 cells, which have been widely used in the motor neuron research, can induce apoptosis (Cheema et al., 2005). Therefore, in the present test, pCK-hSOD1-wild type and pCK-hSOD1-G93A were manufactured by inserting human SOD1 wild type gene (wild type; WT) (NM_000454) and the human SOD1 gene, in which the 93^{rd} amino acid residue was substituted from glycine to alanine, into the BamHI site of the pCK vector, respectively. The following test was carried out to investigate the effect of pCK-HGFX7 in NSC34 cells in which hSOD1-G93A was delivered using the prepared plasmid.

NSC-34 cells were seeded in a 96-well plate such that the cells were suspended at 1×10^4 in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum. The next day, the cells were transfected with pCK, pCK-hSOD1-wild type (WT), and pCK-hSOD1-G93A mutant (G93A) using the lipofectamin LTX reagent (Life technologies, USA). Immediately before the transfection, G93A-transfected cells were treated with the supernatants obtained from 293T cells that were transfected with pCK-HGF728 and pCK-HGFX7 such that the

concentration of HGF protein was 50 ng/㎖. The supernatant obtained by transfecting cells with pCK vector was used as a control.

After culturing for 3 days, the cell growth was confirmed through the treatment with the XTT reagent. The results confirmed that the wild type hSOD1-delivered cells and the pCK vector-delivered cells showed a similar cell growth. However, the G93A mutant hSOD1-delivered cells showed about 85.8% cell growth, compared with the pCK-delivered cells, showing the deterioration in cell growth compared with the wild type hSOD1-delivered cells. However, the pCK-HGFX7 treatment group shows about 92.9% cell growth, indicating the effect of inhibiting the deterioration in cell growth, which is caused by the delivery of G93A mutant hSOD1 (see FIG. 7).

[Gene sequences]

SEQ ID NO: 11: GGC AGA CAG TGA CCA TCT TT

SEQ ID NO: 12: AGT GGA CCT GAG GTT TAT TG

SEQ ID NO: 13: CCA TCA ATC AAA GCC AAG CA

SEQ ID NO: 14: AGC CTT CAC GCA AGT TCA GG

SEQ ID NO: 15: CCA TCA CTG CCA CTC AGA AGA C

SEQ ID NO: 16: TCA TAC TTG GCA GGT TTC TCC

Example 6: Verification of pCK-HGFX7 on grip strength in human mutant SOD1-G93A Tg mouse (Hereinafter, ALS mouse)

As superoxide dismutase 1 (SOD1) mutation has been found to be one of the ALS causes, the ALS mouse model using this gene was developed, and currently, ALS researchers throughout the world have conducted various researches using this animal model. Out of these, B65JL-Tg(SOD1*G93A)1Gur/J (002726), which has been widely used, was selected, and used for tests. The manufacture of the ALS mouse model was requested to Woo Jung BSC (Korea), and the mouse model was used for the present test after

it was verified whether the mutant type SOD1 gene was expressed, through genotyping.

10-week aged ALS mice were divided into 4 mice per group: Tg-pCK, Tg-pCK-HGF728 (pCK-cHGF in PCT/KR03/00548), and Tg-pCK-HGFX7 administration groups. Six mice without Tg were selected and set as a negative control (hereinafter, non-Tg). After the two weeks, the mice of the three test groups, excluding the negative control, were administered with the corresponding plasmid via intramuscular injection. Herein, 50 ㎕ of the corresponding plasmid was administered at 2 ㎍/㎕ to arm triceps muscle, tibial muscle, musculus rectus femoris, and gastrocnemius muscle in left and right, respectively. After two weeks of the administration (14 week age), the grip strength of each mouse was investigated through the behavior test. For the behavior test, the mesh grip strength test was conducted. The mesh grip strength test was used to assess grip strength by placing a mouse on a wire net having lattices at predetermined intervals, overturning the wire net, and then measuring the time while the mouse is suspended from the wire net. This is one of the representative methods for assessing muscular strength of the mouse (Crawley JN, 2008).

As a test result, the non-Tg mice were overturned for an average of about 9 min, but, out of Tg individuals, the mice receiving pCK were suspended and overturned for an average of about 30 s. The individual receiving pCK-HGF728 plasmid showed a slight increased average duration time compared with pCK administration group, and the duration was an average of 49 s. Whereas, the mice receiving pCK-HGFX7 were suspended and overturned for a longer time compared with the mice receiving pCK or pCK-HGF728, and the average duration time was 3 min (see FIG. 8). This shows that pCK-HGFX7

significantly improved the muscular function, including the grip strength, of ALS mice compared with pCK and pCK-HGF728.

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

Claims

1. A pharmaceutical composition for preventing or treating amyotrophic lateral sclerosis, the composition containing, as an active ingredient, two or more isomers of hepatocyte growth factor (HGF) or a polynucleotide encoding the isomers.

2. The composition of claim 1, wherein the two or more isoforms of HGF include full-length HGF (flHGF) and deleted variant HGF (dHGF).

3. The composition of claim 2, wherein the full-length HGF includes the amino acid sequence of SEQ ID NO: 1, and the deleted variant HGF includes the amino acid sequence of SEQ ID NO: 2.

4. The composition of claim 1, wherein the two or more isoforms of HGF are encoded by separate nucleotide sequences.

5. The composition of claim 1, wherein the two or more isoforms of HGF are encoded by a single nucleotide sequence.

6. The composition of claim 1, wherein the polynucleotide is naked DNA, or is contained in a gene delivery system.

7. The composition of claim 6, wherein the gene delivery system is a vector.

8. The composition of claim 7, wherein the vector is a plasmid.

9. The composition of claim 8, wherein the vector is pCK.

10. A method for preventing or treating amyotrophic lateral sclerosis, the method comprising administering, to a mammal, a composition containing, as an active ingredient, two or more isomers of hepatocyte growth factor (HGF) or a polynucleotide encoding the isomers.

11. The method of claim 10, wherein the two or more isoforms of HGF include full-length HGF (flHGF) and deleted variant HGF (dHGF).

12. The method of claim 11, wherein the full-length HGF includes the amino acid sequence of SEQ ID NO: 1, and the deleted variant HGF includes the amino acid sequence of SEQ ID NO: 2.

<110> Viromed Co., Ltd.

<120> Composition for Preventing or Treating Amyotrophic Lateral Sclerosis using Two or More Isoforms of Hepatocyte Growth Factor

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Thr Leu Ile Lys Ile Asp Pro Ala Leu Lys Ile Lys Thr Lys Lys Val
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385                 390                 395                 400

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               405                 410                 415

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        275                 280                 285

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    290                 295                 300

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            580                 585                 590

Asp Leu Pro Asn Tyr Gly Cys Thr Ile Pro Glu Lys Thr Ser Cys Ser
        595                 600                 605

Val Tyr Gly Trp Gly Tyr Thr Gly Leu Ile Asn Tyr Asp Gly Leu Leu
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Arg Val Ala His Leu Tyr Ile Met Gly Asn Glu Lys Cys Ser Gln His
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