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(71) Applicant: **Japan Science and Technology Agency**

**Kawaguchi-shi,
Saitama 332-0012 (JP)**

(72) Inventors:

- **HAMAGUCHI, Michinari**
**Nagoya-shi,
Aichi 4580-846 (JP)**

• **KOKURYO, Toshio**

**Nagoya-shi,
Aichi 4640-015 (JP)**

- **NIMURA, Yuji,**
103 Itopia Shimizugaoka
**Nagoya-shi,
Aichi 4670-038 (JP)**

(74) Representative: **Wilson Gunn**

**Chancery House,
Chancery Lane
London WC2A 1QU (GB)**

(54) **OLIGONUCLEOTIDE INHIBITING TUMOR CELL PROLIFERATION AND METHOD THEREFOR**

(57) The present inventors found that NEK2 kinase (accession number NM_002497) is expressed specifically in tumor cells such as bile duct carcinoma cells and that repression of the expression of NEK2 kinase by the use of RNA interference method led to inhibition of the growth of the tumor cells.

A cytostatic agent for bile duct carcinoma and others containing an oligoribonucleotide comprising a segment

of bases identical or complementary to less than 30 consecutive bases of nucleotide sequence of SEQ ID NO: 1, which includes at least 19 consecutive nucleotides of bases 403-423, 507-527 or 989-1009 of SEQ ID NO: 1 (NEK2 gene), or double-stranded RNA composed of the oligoribonucleotides.

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DescriptionField of the Invention

5 **[0001]** This invention relates to a method for inhibiting the growth of tumor cells by repressing the expression of NEK2 gene and more particularly to a method for repressing the expression of NEK2 gene by RNA interference method by the use of a specific oligoribonucleotide and inhibiting the growth of tumor cells.

Prior Art

10 **[0002]** Signal transduction has been studied as a fundamental mechanism in maintaining cellular function, and abnormality in the pathway of signal transduction has been implicated in various diseases such as cancer. A targeted molecular therapy, whose targeted factor is related to signal transduction, has been paid much attention. Nek2 is a protein with molecular weight of 51,763Da composed of 445 amino acids, whose coding gene is located on 1q32.2-q41, and has serine threonine kinase or leucine zipper motif and PPI binding site at the C-terminal. Nek2 was identified in mammals by homology search of NIMA gene (Never In Mitosis A), which is identified in *Aspergillus* and is related to M phase progression.

15 Nek2 kinase has been studied mainly in relation to cell cycle and chromosome segregation (Reference 3). It is known that Nek2 protein is localized in a centrosome and is highly expressed during late G₂ phase. Therefore, Nek2 might be related to the control of G₂/M phase. Nek2 is functional in division of centrosome in G₂ phase by phosphorylating c-Napl, a physiological substrate, during cell cycle (Reference 4). G₂/M phase during cell cycle is a period of chromosome segregation and cell division as well as division of centrosome. Abnormal Nek2 kinase functional during G₂/M phase could induce aneuploidy by uneven chromosome segregation and is referred to a causal factor of tumorigenesis.

20 It has been tried to control cell cycle using the NIMA kinase, particularly to enhance or to inhibit physiological function during G₂/M phase (Reference 1).

25 Furthermore, double-stranded DNA corresponding to the nucleotide sequence of NEK2 kinase gene (accession number NM_002497) is known to be used as RNAi, which inhibits Nek2 kinase and prevents growth of tumor cells (Reference 2).

30 Reference 1: Japanese Patent Application Public Disclosure No. 2003-144168

Reference 2: US2004/0019003 A1

Reference 3: Cell Growth Differ. 5, 625-635, 1992

Reference 4: EMBO J. 17, 470-481, 1998

Problems to be solved by the Invention

35 **[0003]** The present inventors found that NEK2 kinase (accession number NM_002497) is expressed specifically in tumor cells such as bile duct carcinoma cells and that repression of the expression of NEK2 kinase by the use of RNA interference method led to inhibition of the growth of the tumor cells.

Means to solve the Problems

40 **[0004]** The present inventors found effective RNA sequence in inhibiting the growth of tumor cells such as bile duct carcinoma cells using RNA interference method and accomplished the present invention.

45 More specifically, the present invention is an oligoribonucleotide (1), an oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or a double-stranded RNA comprising the oligoribonucleotides (1) and (2), wherein the oligoribonucleotide (1) corresponds to less than 30, preferably less than 27, and more preferably less than 23 consecutive bases of nucleotide sequence of SEQ ID NO: 1 (NEK2 gene), which includes at least 19 consecutive nucleotides of bases 507-527, 1918-1938, 799-819, 989-1009, 1213-1233, 1835-1855, 403-423, 432-452, 1218-1238, 1445-1465, 1015-1035, 633-653, 1443-1463, 204-224, 511-531, 691-711, 802-822, 1017-1037, 1374-1394, 1377-1397, 1379-1399, 895-915, 977-997, 1126-1146, 1353-1373, 1554-1574, 524-544, 1208-1228 or 600-620, preferably 507-527, 1918-1938, 799-819, 989-1009, 1213-1233, 1835-1855, 403-423, 432-452, 1218-1238, 1445-1465, 1015-1035, 633-653 or 1443-1463, more preferably 507-527, 1918-1938, 799-819, 989-1009, 1213-1233, 1835-1855 or 403-423 of SEQ ID NO: 1.

50 Furthermore, the present invention is a cytostatic agent containing, as an effective component, the oligoribonucleotide (1), the oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or the double-stranded RNA comprising the oligoribonucleotides (1) and (2).

55 **[0005]** Moreover, the present invention is a method for inhibiting growth of tumor cells, comprising introducing, into the tumor cells, the oligoribonucleotide (1), the oligoribonucleotide (2) that is complementary to the oligoribonucleotide

(1) or the double-stranded RNA comprising the oligoribonucleotides (1) and (2).

Still furthermore, the present invention is a kit of growth inhibition of tumor cells containing the oligoribonucleotide (1), the oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or the double-stranded RNA comprising the oligoribonucleotides (1) and (2).

5

Brief Description of the Drawings

[0006]

10 Figure 1 shows the microarray examined the gene expression in three kinds of cholangiocellular carcinoma cells (HuCCT1, TFK1, HuH28).

Figure 2 is the Western blotting showing NEK2 expression in five kinds of cholangiocellular carcinoma cells (HuCCT1, TFK1, HuH28, CCKS1, 293).

15 Figure 3 is the Western blotting showing the repressed expression of NEK2 by each double-stranded RNA in cholangiocellular carcinoma cells (HuCCT1).

Figure 4 shows growth inhibition of cholangiocellular carcinoma cells (HuCCT1) infected with each double-stranded RNA. The ordinate shows absorbance.

Figure 5 are photographs showing the effect of siRNA27 on BalB/c mouse injected with cholangiocellular carcinoma cells (HuCCT1).

20 Figure 6 shows the effect of siRNA27 on tumor volume of BalB/c mouse injected with cholangiocellular carcinoma cells (HuCCT1).

Figure 7 shows the survival benefit of mouse with bile duct carcinoma administered siRNA27.

Detailed Description of the Invention

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[0007] Originally, RNA interference was reported as a method for repressing a specific gene expression by the use of long double-stranded RNA in nematodes. In mammals, long double-stranded RNA induced immunological response and did not induce gene repression. However, it has been demonstrated that small double-stranded RNA (siRNA) induced gene repression in mammals. At present, the size of siRNA mainly used is 19-23 base double-stranded RNA. While more than 30 bases induce immunological response, less than 16 base double-stranded RNA results in increased production of complementary sequence, loses gene specificity for repression, and has a possibility to repress other genes than the target gene.

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[0008] In the present invention, siRNA with the following features is effective:

35

1. The initial two bases are AA,
2. The percent composition of G plus C in the sequence is 42 to 58%.

[0009] The siRNA of NEK2 gene (SEQ ID NO: 1) satisfying the above two features is classified into the following three groups. The first group: siRNA having complementary sequence of consecutive 2 bases and having more than 4 base single-stranded RNA at the 3' end in the secondary structure. The practical example of this group includes the following sequence. The number at the head of the sequence shows the base number in SEQ ID NO: 1 (NEK2 gene).

40

507-527	AAGGAATGCCACAGACGAAGT (No of free bases at the 3' end is 11)
1918-1938	AACCCAGTTAGATGCAATTTG (No of free bases at the 3' end is 7)
45 799-819	AAGAACTCGCTGGGAAAATCA (No of free bases at the 3' end is 6)
989-1009	AAGAGGGCGACAATTAGGAGA (No of free bases at the 3' end is 6)
1213-1233	AACGGAAGTTCCTGTCTCTGG (No of free bases at the 3' end is 6)
1835-1855	AATGACTGAGTGGTATGCTTA (No of free bases at the 3' end is 5)
50 403-423	AAGGAGGGGATCTGGCTAGTG (No of free bases at the 3' end is 4)

[0010] The second group: siRNA having complementary sequence of consecutive 2 bases and having less than three base single-stranded RNA at the 3' end in the secondary structure.

The practical example of this group includes the following sequence. The number at the head of the sequence shows the base number in SEQ ID NO: 1 (NEK2 gene).

55

432-452	AAGGGAACCAAGGAAAGGCAA (No of free bases at the 3' end is 3)
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(continued)

5
1218-1238 AAGTTCCTGTCTCTGGCAAGT (No of free bases at the 3' end is 2)
1445-1465 AAGCAGACAGATCCTGGGCAT (No of free bases at the 3' end is 2)
1015-1035 AAAAAATCGCAGGATTCCAGCC (No of free bases at the 3' end is 1)
633-653 AACCATGACACGAGTTTTGCA (No of free bases at the 3' end is 0)
1443-1463 AAAAGCAGACAGATCCTGGGC (No of free bases at the 3' end is 0)

10 **[0011]** The third group: siRNA having complementary sequence of consecutive more than 3 bases.

The practical example of this group includes the following sequence. The number at the head of the sequence shows the base number in SEQ ID NO: 1 (NEK2 gene). 3 base complementary sequence:

15
204-224 AAGATCCGGAGGAAGAGTGAT
511-531 AATGCCACAGACGAAAGTGATG
691-711 AACAAATGAATCGCATGTCCT
802-822 AACTCGCTGGGAAAATCAGAG
1017-1037 AAATCGCAGGATTCCAGCCCT
1374-1394 AAGAAAAGGCTTCACGCTGCC
20 1377-1397 AAAAGGCTTCACGCTGCCAG
1379-1399 AAGGCTTCACGCTGCCAGCT

4 base complementary sequence:

25
895-915 AAAGGATTACCATCGACCTTC
977-997 AAATCTTGAGAGAAGAGGGCG
1126-1146 AACAGGAGCTTTGTGTTCTGTG
30 1353-1373 AAGTCCAAGTGCAAGGACCTG
1554-1574 AATACTTGGCCCCATGAGCCA

5 base complementary sequence:

35 524-544 AAGTGATGGTGGTCATACCGT

6 base complementary sequence:

40 1208-1228 AAAGGAACGGAAGTTCCTGTC

8 base complementary sequence:

45 600-620 AAGCTTGGAGACTTTGGGCTA

50 **[0012]** The first group is the most effective, followed in order by the second and the third group. Since reassociation of the complementary sequences in a molecule may leave free bases at the 3' end, it has been interpreted that the more the number of free bases is, the more effective the activity of repression of NEK2 kinase is by RNA interference. The siRNA of the present invention is an oligoribonucleotide (1), an oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or a double-stranded RNA comprising the oligoribonucleotides (1) and (2), wherein the oligoribonucleotide (1) corresponds to less than 30, preferably less than 27, and more preferably less than 23 consecutive bases of nucleotide sequence of SEQ ID NO: 1, which includes at least 19 consecutive nucleotides of bases in SEQ ID NO: 1.

55 **[0013]** Furthermore, it is reported in the Reference 2 that double-stranded RNA corresponding to a part of the sequence of NEK2 kinase (accession number NM_002497) is used as RNAi inhibiting Nek2 kinase and preventing the growth of tumor cells. However, the sequence described in the reference is exhaustive. Although it is described in the example that the sequence of SEQ ID NO: 3 to SEQ ID NO: 12, particularly that of SEQ ID NO:12 is effective, the siRNA of the present invention is selected based on completely different point of view and is different from the sequence of the previous

reference. Actually, RNA interference effect of SEQ ID NO: 3 to SEQ ID NO: 12, in which SEQ ID NO: 12 corresponds to siRNA528 in the Example 1 of the present patent application, is extremely inferior to that of the present invention.

[0014] Although RNA fragment used in the present invention could be sense or antisense RNA of the target RNA, these single-stranded RNA might be easily degraded by RNase and be less effective. Therefore, double-stranded RNA composed of the RNA is preferably used. The double-stranded RNA is usually prepared by hybridization of two single-stranded RNA (i.e. sense and antisense RNA) prepared separately.

The oligoribonucleotide "corresponding to" a specific sequence of NEK2 gene means the RNA complementary to a part of mRNA generated after transcription of NEK2 gene, in which the part of mRNA corresponds to the specific sequence of NEK2 gene. Practically, the oligoribonucleotide is obtained by replacing T of the specific DNA sequence of NEK2 gene with U.

[0015] The target cells of the present invention are tumor cells of human et al. These tumor cells include biliary tract carcinoma (cholangiocellular carcinoma, gall bladder cancer), breast cancer, pancreatic cancer, esophagus cancer, gastric cancer, colorectal carcinoma, hepatocellular carcinoma, lung cancer, larynx cancer, pharyngeal cancer, thyroid cancer, uterine cancer, ovarian cancer, renal carcinoma, prostate carcinoma, bladder carcinoma, malignant melanoma, brain tumor. Cytostatic agent of tumor cells may include an oligoribonucleotide, the complementary oligoribonucleotide, or double-stranded RNA composed of these oligoribonucleotides as an effective component, and furthermore may include antisenses, DNA enzymes, peptides, or neutralising antibodies.

[0016] Furthermore, the cytostatic agent of tumor cells could be combination with other known cytostatic agents and others. The cytostatic agent of tumor cells of the present invention may be a form of kit containing other agents as described, or may contain such pharmaceutically acceptable medium as sterilized isotonic saline, preservatives, buffer and others.

Moreover, the cytostatic agent of tumor cells of the present invention may provide such kits as injection kit to administer the formulated the cytostatic agent after mixing with diluent and tablet kit to administer individual formulated tablet.

There is no restriction of the method for introducing RNA fragment into cells and the method includes calcium phosphate method, microinjection method, protoplast fusion method, electroporation, and the use of virus vector. Commercially available transfection reagent based on liposome is conveniently used.

[0017] The examples of administration of siRNA of the present invention includes the following methods:

(1) As a preoperative administration for biliary tract carcinoma (cholangiocellular carcinoma, gall bladder cancer), (1-1) siRNA dissolved in saline is administrated into hepatic artery under angiography. (1-2) siRNA dissolved in saline or cell matrix^R (Nitta Geratin) is administrated directly into bile duct via percutaneous transhepatic biliary drainage (PTCD) tube, or administrated directly into tumor by ultrasound guided endermic paracentesis.

(2) For cancerous peritonitis (peritoneal dissemination) derived from biliary tract carcinoma (cholangiocellular carcinoma, gall bladder cancer), siRNA dissolved in saline or cellmatrix^R (Nitta Geratin) is administered directly into peritoneal cavity by abdominal operation or administered endermically into peritoneal cavity under ultrasound guidance.

(3) For inoperable patients with biliary stenosis due to biliary tract carcinoma (cholangiocellular carcinoma, gall bladder cancer), siRNA dissolved in saline or cellmatrix^R (Nitta Geratin) is administered directly into bile duct via percutaneous transhepatic biliary drainage (PTCD) tube.

[0018] The following Examples illustrate the present invention, but it is not intended to limit the scope of the present invention. In the following Example, five kinds of cholangiocellular carcinoma cell lines (HuCCT1, TFK1, HuH28, CCKS1, 293) were used, and HuCCT1, TFK1 and HuH28 are obtained from Institute of Development, Aging and Cancer, Tohoku University; CCKS1 (cell line established from bile duct carcinoma) is from Pathology Department, Kanazawa University; 293 (cell line established from faetal kidney) is from RIKEN.

Test Example 1

[0019] In this Example, exhaustive gene analysis on three kinds of cholangiocellular carcinoma cell lines (HuCCT1, TFK1, HuH28) and four kinds of clinical samples was performed and the expression of NEK2 was investigated.

Cholangiocellular carcinoma cells are cultured in 10 cm Petri dish up to 80 % confluent. The culture was washed two times with PBS, homogenized with 26G injection needle after addition of 1 ml TRIzolTM (LIFE TECHNOLOGIES) in 10 cm dish and was allowed to rest at room temperature for 30 min. RNA was recovered by extraction with chloroform and by precipitation in isopropyl alcohol according to the manual of TRIzolTM.

RNA was added with 1ml of 75% ethanol, centrifuged at 15000rpm for 10 min at 4°C and recovered as a precipitation. RNA was dissolved in 100 μ l RNA free water on ice. mRNA was purified by the use of μ MACS mRNA isolation kitTM (Millenyl Biotec). Dissolved RNA was added with 200 μ l Lysis/Binding Buffer according to the manual of μ MACS mRNA isolation kitTM. It was mixed well, heated at 68°C for 3 min and allowed to rest on ice for 10 min.

Furthermore, 50 μ l Oligo (dt) Microbeads was added to the dissolved RNA solution. mRNA was extracted with MACS column Type μ . The yield of RNA was determined by the measurement of absorption at 260 nm and 280 nm. mRNA was precipitated by ethaninmate and stored at -80°C.

[0020] In contrast, 1 g excised tissue (normal liver or bile duct carcinoma) was frozen in liquid nitrogen, crushed, added with 1 ml TRIzol™ homogenized with 26G injection needle and was allowed to rest at room temperature for 30 min. RNA extraction and mRNA purification was performed as above described.

[0021] ³²P labeled cDNA was prepared from 5 μ g mRNA, DNA primer and α -³²PdATP by the use of reverse transcriptase according to the manual of Atlas Human Cancer 1.2 Array™ (CLONTECH). Total five μ g mRNA composed of each 1 μ g mRNA extracted from normal liver tissue of 5 cases was used as a control. Hybridization was performed for 9 hr at 68°C, after addition of ³²P labeled cDNA onto array membrane after prehybridization. The array membrane was washed with solution 1 for 30 min for three times at 68°C and with solution 2 for 30 min for two times at 68°C. The array membrane was packed in plastic bag and exposed to an image plate at room temperature for 48 hrs. The isotope signal was detected by an image analyzer. For individual gene, total ionizing radiation dose was measured as signal strength (PSL: PSL= α DT, D: radiation dose of RI labeled sample on image plate, T: exposure time, α : constant) and radiation dose per area was calculated based on an image area. The result was shown in Fig. 1.

[0022] After the correction of back ground of measured radiation dose per area, correction by β -actin was performed to normalize the expression of house keeping gene among various array membranes. The ratio of signal strength of cholangiocellular carcinoma cells against that of control was expressed by log₂. More than 1, which means the ratio of expression is more than two times, is interpreted as enhanced expression and less than -1, which means that the ratio of expression is less than 1/2, is interpreted as repressed expression.

For NEK2, the 6th gene from the above, the expression was more than 1 for all cell lines and clinical samples, i.e. the expression is enhanced. Particularly, for HuCCT1, the remarkably enhanced expression is exhibited.

Test Example 2

[0023] In this Example, the expression of NEK2 was examined for 5 kinds of cholangiocellular carcinoma cells (HuCCT1, TFK1, HuH28, CCKS1, 293) by Western blotting.

First of all, cell lysate of cholangiocellular carcinoma cells was prepared. Each of five kinds of cholangiocellular carcinoma cells was cultured in 10 ml Petri dish up to 80% confluent. The cultures were washed with PBS for two times, added with 300 μ l sample buffer, homogenized with 21G injection needle and heated at 96°C for 3 min. Then, a separation gel was prepared. 8.52 ml sterilized distilled water, 11.1 ml of 1 M Tris -HCl (pH 8.8), 300 μ l of 10 % SDS, 10 ml bis-acrilamid, 300 μ l APS, and 25 μ l TEMED were mixed and allowed to rest at room temperature for 30 min.

Later, a stacking gel was prepared. 4.22 ml sterilized distilled water, 0.75 ml of 1 M Tris -HCl (pH 6.8), 60 μ l of 10 % SDS, 0.9 ml bis-acrilamid, 60 μ l APS, and 10 μ l TEMED were mixed, overlaid on a separation gel and allowed to rest at room temperature for 15 min.

[0024] After that, 20 μ l of each lysate together with makers was electrophoresed for 60 min under CC40A. After the electrophoresis, a transfer membrane was overlaid on the gel, energized at 15 V for 45 min, and transferred the proteins on the gel to the transfer membrane.

[0025] In the meantime, 5 % skim milk solution was prepared by adding 50 ml 1 x TBS-T to 2.5 g skim milk. Blocking solution was prepared by putting transfer membrane into 5% skim milk solution and by warming at 37°C for 60 min with constant shaking.

[0026] Then, the first antibody solution diluted to 500 fold was prepared by the addition of 500 μ l of 5% skim milk to 1 μ l first antibody (NEK2: Transduction Laboratories or β -actin: SIGMA) and by mixing. The transfer membrane was added to the first antibody solution and warmed at 37°C for 60 min with constant shaking. The transfer membrane was washed for three times with 1 x TBS-T at room temperature for 10 min.

[0027] The second antibody solution diluted to 4000 fold was prepared by the addition of 2 ml of 5% skim milk to 0.5 μ l second antibody (Goat F(ab') anti Mouse Ig's HRP conjugated: BIOSOURCE) and by mixing. The transfer membrane was added to the second antibody solution and warmed at 37°C for 60 min with constant shaking. The transfer membrane was washed for three times with 1 x TBS-T at room temperature for 10 min.

[0028] ECL™ reaction solution was prepared by the mixture of 500 μ l ECLA solution with 500 μ l B solution according to a manual of ECL™ solution (PerkinElmer Life Science). Transfer membrane was enclosed in a plastic bag and was added with ECL reaction solution. The transfer membrane was exposed to Roentgen film for two min at room temperature in a dark room. The Roentgen film was developed and NEK2 was detected by chemiluminescence.

The result is shown in Fig.2. NEK2 is composed of NEK2A and NEK2B, wherein the C-terminal of NEK2B is spliced from NEK2A. The level of expression was compared based on the ratio of NEK2 concentration to the data of β -actin used as a control and HuCCT1 cells were found to express NEK2 the most highly.

In the following Examples, cholangiocellular carcinoma cells (HuCCT1) were used because they expressed the most highly, are transplantable into nude mouse and proliferated the most rapidly.

<Example 1>

[0029] In the Example, repressed expression of NEK2 in cholangiocellular carcinoma cells (HuCCT1) was examined with 5 kinds of double-stranded RNA by the use of Western blotting. The following 5 kinds of siRNA were constructed and prepared.

(1) siRNA 65 : Sense strand siRNA (GAGGGCGACAAUUAGGAGAtt) and antisense strand siRNA (UCUCCUAA-UUGUCGCCCUCtt) corresponding to AAGAGGGGCGACAATTAGGAGA (SEQ ID NO: 6) were prepared and used after hybridization.

(2) siRNA 27: Sense strand siRNA (GGAAUGCCACAGACGAAGUtt) and antisense strand siRNA (ACUUCGUCU-GUGGCAUUCctt) corresponding to AAGGAATGCCACAGACGAAGT (SEQ ID NO: 7) were prepared and used after hybridization.

(3) siRNA 19 :Sense strand siRNA (GGAGGGGAUCUGGCUAGUGtt) and antisense strand siRNA (CACUAGCCA-GAUCCCCUCctt) corresponding to AAGGAGGGGATCTGGCTAGTG (SEQ ID NO: 8) were prepared and used after hybridization.

(4) siRNA65+27:The equal amount of (1) and (2) were mixed and used.

(5) siRNA128 : Sense strand siRNA and antisense strand siRNA corresponding to AGAAAAATAATATTAGGAAAA (SEQ ID NO: 9) were prepared and used after hybridization.

(6) siRNA528 : Sense strand siRNA and antisense strand siRNA corresponding to CCTGGATGGCAAG-CAAACGTC (SEQ ID NO: 10) were prepared and used after hybridization.

(7) Luciferase GL2 siRNA: Commercial product (DHARMACON) was used as a control. This is siRNA against Luciferase, which is not present in human body. It affects neither to the expression of NEK2 nor to cell proliferation.

The concentration of each siRNA was prepared at 20 μ M and used for the example.

[0030] On the other hand, cholangiocellular carcinoma cells (HuCCT1) were cultured in 3.5 cm Petri dish until 60% confluent. The culture was washed two times with the same medium without serum and added with 800 μ l of the medium without serum.

4 μ l of each 20 μ M siRNA and 96 ml medium were mixed. At the same time, 20 μ l GenePOTER™ (Gene Therapy System) and 80 μ l medium were mixed. 200 μ l siRNA GenePOTER™ mixture was prepared by their mixing and allowed to rest for 30 min at room temperature. The above siRNA GenePOTER™ was added to the 3.5 cm culture dish containing cholangiocellular carcinoma cells and medium without serum. It was incubated for 3 hr at 37°C in CO₂ incubator. Then, 1ml of medium containing 20% serum was added to the 3.5 cm dish containing siRNA Gene POTER™ mixture. It was incubated in CO₂ incubator at 37°C for 72 hr. The expression of a protein was examined by the Western blotting described in the Test Example 2.

The result is shown in Fig.3. At 72 hr time point, siRNA27 and siRNA19 show the similar repression of NEK2 protein expression. On the other hand, siRNA128 and siRNA528 show similar repression to the control and show no repression effect of NEK2 protein expression.

[0031] In addition, siRNA65 show little repression effect at 72 hr time point, however it shows certain repression effect at one week time point (the result is not shown). The action mechanism of siRNA27 may be different from that of siRNA19 and siRNA65.

<Example 2>

[0032] In this Example, proliferation assay was performed for cholangiocellular carcinoma cells (HuCCT1) infected with double-stranded RNA used in Example 1. As a control, the result by the use of the cell lines infected with only GenePORTER™ is shown.

Cholangiocellular carcinoma cells (HuCCT1) were infected with five kinds of double-stranded RNA according to the method shown in Example 1.

The culture was washed with PBS for two times, added with 1ml of 0.1% trypsin/EDTA and incubated in a CO₂ incubator at 37°C for 10 min. The cell number was counted and the cell suspension in the medium was prepared at 1 x 10⁶/ml.

Each group is composed of 10 wells in 96 well plate and each well was seeded with 50 μ l cells. The plate was incubated in a CO₂ incubator at 37°C for 24 hr. Each well was added with 50 μ l medium and 10 μ l TetraColor ONE™ (SEIKAGAKU CORPORATION). It was incubated in a CO₂ incubator at 37°C for 3 hr. The absorbance at 450 nm was measured by a microplate reader.

The result is shown in Fig. 4. As in the case of Example 1, siRNA27 and siRNA19 show similar repression effect of expression of NEK2 protein and siRNA 65 shows slight repression effect. In contrast, siRNA 128 and siRNA 528 show similar repression to the control and show no repression effect of NEK2 protein expression.

<Example 3>

[0033] In this Example, effect of siRNA was examined for BalB/c mice inoculated with cholangiocellular carcinoma cells (HuCCT1).

5 Cholangiocellular carcinoma cells (HuCCT1) were cultured in 15 cm Petri dish until 80% confluent. The culture was washed with PBS for two times, added with 1 ml of 0.1% trypsin/EDTA solution, incubated in a CO₂ incubator at 37°C for 10 min. The culture was washed with PBS for two times. Cell number was counted and the cells were suspended in Hanks™ solution (LIFE TECHNOLOGIES) at 1 x 10⁷/100 μl. Cholangiocellular carcinoma cells (HuCCT1) were injected subcutaneously into a right thigh of an 8 w.o. BalB/c mouse at 1 x 10⁷ cells / 100 μl. The mouse was farmed for a month after the injection.

10 100 μl of 20 μM siRNA 27 was directly injected subcutaneously into tumor in the right thigh. Injection was performed once a week for total three times. After one month of the third siRNA 27 injection, the tumor was exsected and the volume of the tumor was measured and is shown in Fig. 5. Figure 6 shows that size of the tumor decreases according to the administration of siRNA 27.

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<Example 4>

[0034] Bile duct carcinoma was injected into mouse peritoneal cavity. After peritoneal dissemination, siRNA 27 was administrated and the survival benefit of the mouse was examined. The result is shown in Fig. 7. Unquestionably, survival benefit was observed.

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[0035] The oligoribonucleotide of the present invention could be used as a cytostatic agent of tumor cells. Furthermore, the oligoribonucleotide may possibly inhibit growth of inflammatory cells and could be used as preventive medicine for keloidosis, which is induced at the time of operation wound healing.

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Claims

1. An oligoribonucleotide (1), an oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or a double-stranded RNA comprising the oligoribonucleotides (1) and (2), wherein the oligoribonucleotide (1) corresponds to less than 30 consecutive bases of nucleotide sequence of SEQ ID NO: 1 (NEK2 gene), which includes at least 19 consecutive nucleotides of bases 507-527, 1918-1938, 799-819, 989-1009, 1213-1233, 1835-1855, 403-423, 432-452, 1218-1238, 1445-1465, 1015-1035, 633-653, 1443-1463, 204-224, 511-531, 691-711, 802-822, 1017-1037, 1374-1394, 1377-1397, 1379-1399, 895-915, 977-997, 1126-1146, 1353-1373, 1554-1574, 524-544, 1208-1228 or 600-620 of SEQ ID NO: 1.
2. An oligoribonucleotide (1), an oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or a double-stranded RNA comprising the oligoribonucleotides (1) and (2), wherein the oligoribonucleotide (1) corresponds to less than 30 consecutive bases of nucleotide sequence of SEQ ID NO: 1 (NEK2 gene), which includes at least 19 consecutive nucleotides of bases 507-527, 1918-1938, 799-819, 989-1009, 1213-1233, 1835-1855, 403-423, 432-452, 1218-1238, 1445-1465, 1015-1035, 633-653 or 1443-1463 of SEQ ID NO: 1.
3. An oligoribonucleotide (1), an oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or a double-stranded RNA comprising the oligoribonucleotides (1) and (2), wherein the oligoribonucleotide (1) corresponds to less than 30 consecutive bases of nucleotide sequence of SEQ ID NO: 1 (NEK2 gene), which includes at least 19 consecutive nucleotides of bases 507-527, 1918-1938, 799-819, 989-1009, 1213-1233, 1835-1855 or 403-423 of SEQ ID NO: 1.
4. An oligoribonucleotide (1), an oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or a double-stranded RNA comprising the oligoribonucleotides (1) and (2), wherein the oligoribonucleotide (1) corresponds to less than 30 consecutive bases of nucleotide sequence of SEQ ID NO: 1 (NEK2 gene), which includes at least 19 consecutive nucleotides of bases 403-423, 507-527 or 989-1009 of SEQ ID NO: 1.
5. A cytostatic agent containing, as an effective component, the oligoribonucleotide (1), the oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or the double-stranded RNA comprising the oligoribonucleotides (1) and (2) of any one of claims 1 to 4.
6. A method for inhibiting growth of tumor cells, comprising introducing, into the tumor cells, the oligoribonucleotide (1), the oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or the double-stranded RNA

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comprising the oligoribonucleotides (1) and (2) of any one of claims 1 to 4.

7. A kit of growth inhibition of tumor cells containing the oligoribonucleotide (1), the oligoribonucleotide (2) that is complementary to the oligoribonucleotide (1) or the double-stranded RNA comprising the oligoribonucleotides (1) and (2) of any one of claims 1 to 4.

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Figure 1

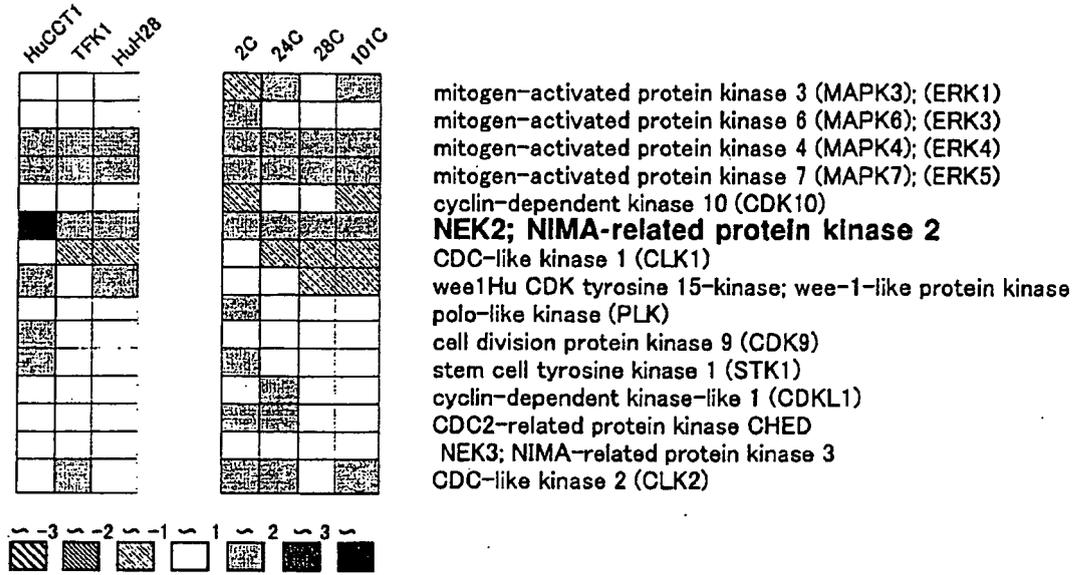


Figure 2

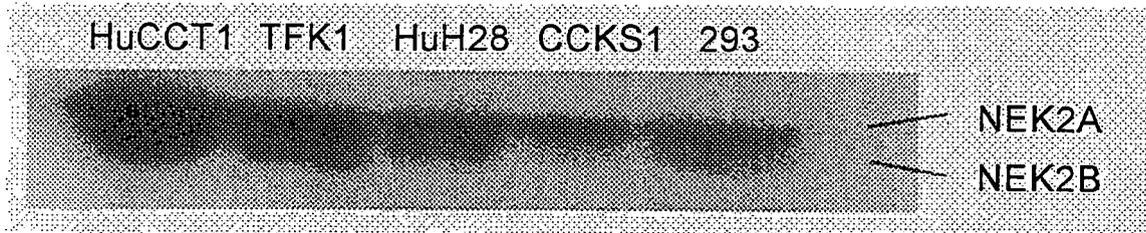


Figure 3

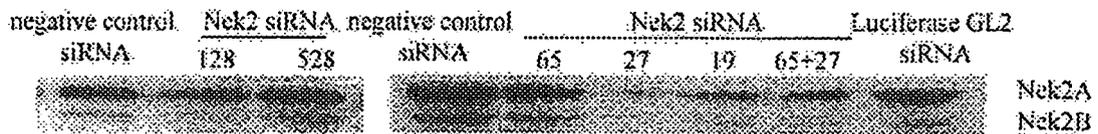


Figure 4

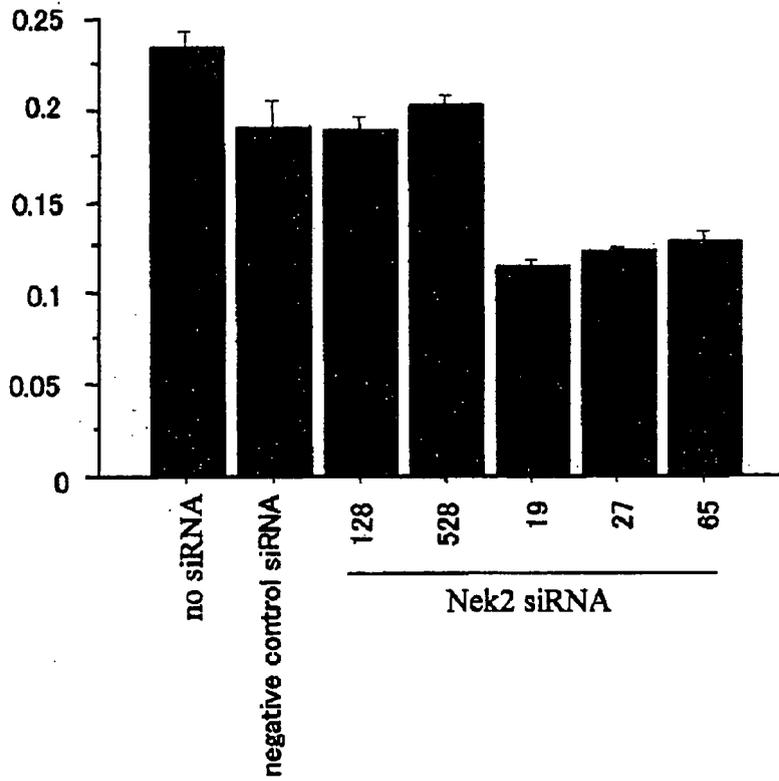


Figure 5

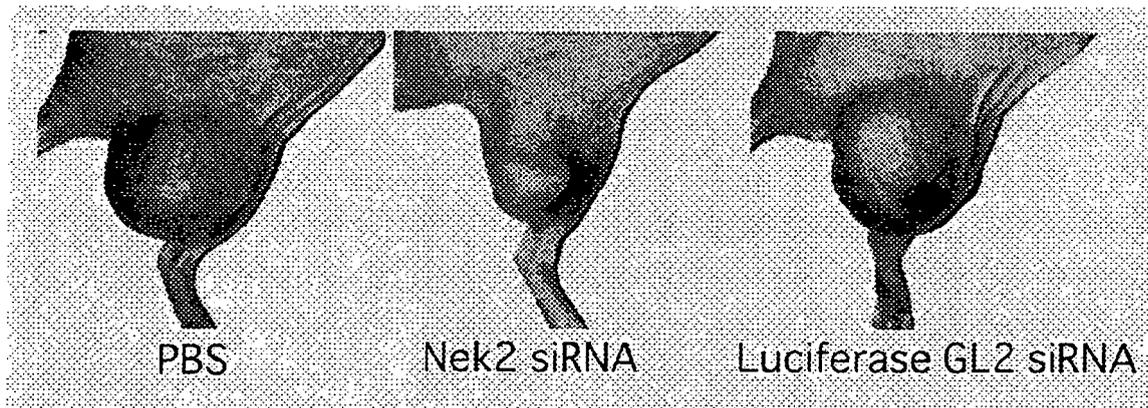


Figure 6

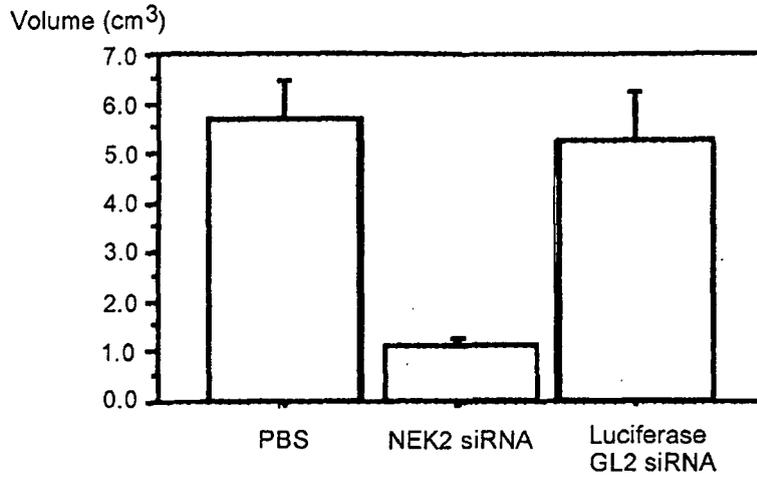
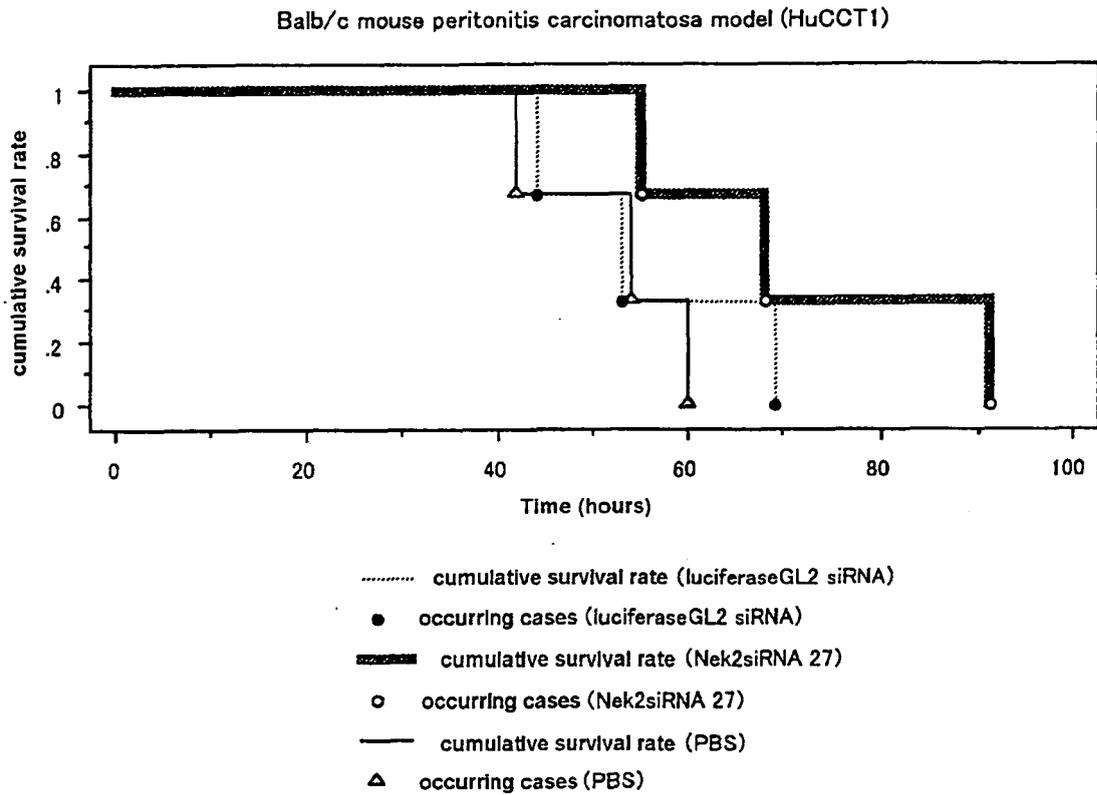


Figure 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2005/011227

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl ⁷ C12N15/09, A61K31/7105, 31/713, 48/00, A61P35/00, C07H21/02		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) Int.Cl ⁷ C12N15/09, A61K31/7105, 31/713, 48/00, A61P35/00, C07H21/02		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA (STN), REGISTRY (STN), BIOSIS/WPI (DIALOG), JSTPlus (JOIS)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/0019003 A1 (Chiron Corp.), 29 January, 2004 (29.01.04), Full text & WO 03/062197 A2	1-5, 7
A	Fry A.M. et al., A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators, EMBO J., 1998, Vol.17, No.2, pages 470-81	1-5, 7
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 22 August, 2005 (22.08.05)		Date of mailing of the international search report 06 September, 2005 (06.09.05)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2005/011227

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 6
because they relate to subject matter not required to be searched by this Authority, namely:
It involves to methods for treatment of the human body by therapy.
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(See extra sheet.)

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
(See extra sheet.)

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2005/011227

Continuation of Box No.III of continuation of first sheet(2)

The matter common to claims 1 to 7 resides in an oligoribonucleotide corresponding to a base sequence comprising from 19 to 30 consecutive bases in the NEK2 gene, an oligoribonucleotide complementary thereto, or a double-stranded RNA comprising the same.

As the results of the search, however, it is clarified that the above matter is not novel because of being described in document US 2004/0019003 A1 (Chiron Corp.), 29 January, 2004 (29.01.04).

Consequently, the above matter falls within the category of prior art and, therefore, the common matter cannot be regarded as a special technical feature in the meaning within the second sentence of PCT Rule 13.2.

Accordingly, there is no matter common to all claims.

Since there is no common matter seemingly being a special technical feature in the meaning within the second sentence of PCT Rule 13.2, no technical relevancy can be found among these inventions differing from each other in the meaning within PCT Rule 13.

Such being the case, it is obvious that claims 1 to 7 do not comply with the requirement of unity of invention.

Continuation of Box No.III-4 of continuation of first sheet(2)

In claims 1 to 7, the parts relating to an oligoribonucleotide corresponding to a base sequence of 30 or less consecutive bases at least containing the 19 consecutive bases of base nos. 507 to 527 in the base sequence of SEQ ID NO:1, an oligoribonucleotide complementary thereto, or a double-stranded RNA comprising the same.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- JP 2003144168 A [0003]
- US 20040019003 A1 [0003]

Non-patent literature cited in the description

- *Cell Growth Differ.*, 1992, vol. 5, 625-635 [0003]
- *EMBO J.*, 1998, vol. 17, 470-481 [0003]