NOVEL NUCLEOTIDE SEQUENCES

FIELD OF THE INVENTION:

The present invention relates to the field of cancer therapy. More specifically, the invention relates to use of certain nucleotide sequences for the treatment of cancer.

BACKGROUND OF THE INVENTION:

RNA interference (RNAi) is now an umbrella term referring to post-transcriptional gene silencing mediated by either degradation or translation arrest of target RNA. This process is initiated by double-stranded RNA with sequence homology driving specificity.

RNA interference (RNAi) is an evolutionarily conserved post-transcriptional gene silencing (PTGS) mechanism mediated by double-stranded RNA (dsRNA).The dsRNA is processed into small duplex RNA molecules of approximately 21-22 nucleotides (nts) termed small interfering RNAs (siRNAs) by a RNase III enzyme called Dicer. Interaction of siRNAs with a multi-protein complex, termed the RNA-induced silencing complex (RISC), results in sequence specific association of the activated RISC complex with the cognate RNA transcript. This interaction leads to sequence-specific cleavage of the target transcript.

Originally discovered in *Caenorhabditis elegans*, the study of RNAi in mammalian cells has blossomed in the last couple of years with the discovery that introduction of siRNA molecules directly into somatic mammalian cells circumvents the non-specific response vertebrate cells have against larger dsRNA molecules. Emerging as a powerful tool for reverse genetic analysis, RNAi is rapidly being applied to study the function of many genes associated with human disease, in particular those associated with oncogenesis and infectious disease. Use of siRNA as a tool is advancing in almost every field of biomedical research, but some of the most dynamic and exciting applications of siRNA are in cancer research.

Almost all human cancers have accumulated multiple genetic lesions including oncogenes. It is often unknown whether an oncogene is continuously required for tumorigenesis. Furthermore, it is very difficult to target an essential oncogene with drugs

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without affecting the corresponding nonmutated protooncogene or related factors. RNA interference and the application of small interfering RNAs in mammalian cell culture provide new tools to examine the role of oncogenes in tumor development.

The Applicant has recently cloned a testis specific gene SPAG9 localized on human chromosome 17. It contains coiled coil domains and a leucine zipper motif encoding a protein consisting of 766 amino acids; and has been assigned to UniGene cluster Hs. 129872. Functional analysis of SPAG9 revealed that SPAG9 may have role in one or more events leading to fertilization. Southern hybridization studies suggested that human genome contains single copy of SPAG9 gene having 19 exons. The exons sequence length of SPAG9 varies from 39 to 333. The Applicant sequenced SPAG9 (CAA62987) gene the same bears SEQ ID 17 which encodes the polypeptide (766 aa) and the same bears SEQ ID 18.

Further, based on the above and upon further investigations found that the SPAG9 mRNA is expressed exclusively in normal testis tissue whereas SPAG9 is expressed in a majority of tumors (cancer) and transformed cell lines namely: testis, kidney, uterus, nervous tissue, eye, pituitary, colon, skin, lung, placenta, stomach, urinary bladder, leukopheresis, breast, vulva, pharynx, placenta, bone, prostate and liver.

There is increasing evidence for an immune response to cancer in humans, as demonstrated in part by the identification of autoantibodies against a number of intracellular and surface antigens detectable in sera from patients with different cancer types. The generation of antibodies against SPAG9 in tissues other than testis made the applicant investigate this aspect further and now, the Applicant has now developed novel sequences that are capable of targeting SPAG9 in cancerous tissues.

DESCRIPTION OF THE INVENTION:

Accordingly, in one aspect the invention provides novel nucleotide sequences which are capable of downregulating or interfering with the SPAG9 mRNA which is found to be expressed exclusively in normal testis tissue although SPAG9 is expressed in a majority of tumors (cancer) and transformed cell lines namely: testis, kidney, uterus,

nervous tissue, eye, pituitary, colon, skin, lung, placenta, stomach, urinary bladder, leukopheresis, breast, prostate, vulva, pharynx, placenta, bone and liver.

Thus, the invention provides small interfering ribonucleic acid (siRNA) for inhibiting the expression of protein encoded by SEQ ID 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 in a cell, wherein the siRNA comprises at least 2 sequences that are complementary to each other and wherein a sense strand comprises a first sequence and an anti-sense strand comprises a second sequence comprising a region of complementarity, which is substantially complementary to at least a part of an mRNA encoding a polynucleotide sequence selected from SEQ ID 17.

Some of said novel nucleotide sequences are depicted in Table 1 herebelow.

Table 1:

S.No.	Target sequence for sirRNA	Region	Start
1	AGATCTCAGTGGATATAAA	ORF	638
2.	ACAGCTCATAGTAGAATTA	ORF	186
3.	CAAGGCGGATCTAAAGCTA	ORF	378
4.	GTTACAGATGCGCCAAATA	ORF	483
5.	AGCTCATAGTAGAATTAGA	ORF	188
6.	GGAGCAGATTTACTAGGAA	ORF	771
7.	TTACTCCGTCCGTCAAGAA	ORF	1327
8.	ACAGCTCATAGTAGAATTA	ORF	186
9.	AGAACGCCCTATATCATTA	ORF	209
10.	AGCTCATAGTAGAATTAGA	ORF	188
11.	TTACTCCGTCCGTCAAGAA	ORF	1327
12.	AGAAGCAACTGAAGCTACA	ORF	2240
13.	GTGTATCAGTCGAGGTATA	ORF	2373
14.	ATCAGTCGAGGTATAATAA	ORF	2377
15.	TCAGTCGAGGTATAATAAT	ORF	2378
16.	ATAATGGGTCATCAACTTA	ORF	2392

In another aspect, the present invention also provides compositions useful for inhibiting cancerous cell proliferation. Such compositions may preferably comprise a small interfering ribonucleic acid (siRNA) for inhibiting the expression of protein encoded by SEQ ID 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 in a cell, wherein the siRNA comprises at least 2 sequences that are complementary to each other and wherein a sense strand comprises a first sequence and an anti-sense

strand comprises a second sequence comprising a region of complementarity, which is substantially complementary to at least a part of an mRNA encoding a polynucleotide sequence selected from SEQ ID 17, together with an appropriate cellular uptake-enhancing peptide segment or agent. Also included in the invention are compositions comprising expression vectors containing the said nucleotides, including nucleic acids encoding sequence ID 1-16.

In one aspect, the invention provides a novel method of inhibiting cellular proliferation of cancer cells which method comprises the step of delivering to the cell a composition comprising a nucleotide selected from SEQ IDs 1 to 16. The said nucleotide sequences may be preferably complexed with a cellular uptake-enhancing agent, and may be delivered in an amount and under conditions sufficient to enter the cell, thereby inhibiting cancerous cell growth/ proliferation.

In yet another aspect, the invention provides a novel method of promoting apoptosis which method comprises the step of delivering a composition comprising small interfering ribonucleic acid (siRNA) for inhibiting the expression of protein encoded by SEQ ID 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 in a cell, wherein the siRNA comprises at least 2 sequences that are complementary to each other and wherein a sense strand comprises a first sequence and an anti-sense strand comprises a second sequence comprising a region of complementarity, which is substantially complementary to at least a part of an mRNA encoding a polynucleotide sequence selected from SEQ ID 17, together with an appropriate cellular uptake-enhancing peptide segment or agent. The said nucleotide sequences may be preferably complexed with a cellular uptake-enhancing agent, and may be delivered in an amount and under conditions sufficient to enter the cell, and cause apoptosis.

As is known, gene silencing by RNA interference (RNAi) operates at the level of mRNA that is targeted for destruction with exquisite sequence specificity. The scheme is shown in Figure 1A. In principle, any disease-related mRNA sequence is a putative target for RNAi-based therapeutics. To develop this therapeutic potential, it is necessary to develop ways of inducing RNAi by clinically acceptable delivery procedures.

By preventing translational expression of at least part of the protein encoded by SEQ ID 18 or an isoform thereof or expression of polypeptide comprising the said SEQ ID 18. The sequences are useful, in accordance with the inventive method, to prevent expression of SPAG9 protein or proteins produced by polynucleotide sequences comprising SEQ ID 17 and hence cancer cell growth/ proliferation.

Thus the novel sequences of the invention that can be delivered to mammalian cells and consequently down regulate or block expression of protein encoded by SEQ ID 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18.

Thus, in another aspect, the invention provides a method of using siRNAs capable of recognizing any of SEQ ID 1 to 16 for inhibiting cellular growth of proliferation of cancerous tissues by delivery of a therapeutically effective amount of the said siRNAs to a subject in need thereof.

The invention is now illustrated by the following examples and drawings which are only illustrative and not meant to restrict the scope of the invention in any manner. The following accompanying drawings are appended:

Figure 1A is a schematic representation of siRNA mediated gene silencing.

Figure 1B represents expression of protein comprising SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 in human lung cancer (A549) cell line.

Figure 2 is a Western Blot analysis of protein expression in human lung cancer (A549) cell lysate.

Figure 3 is an indirect immunofluorescence analysis of human lung cancer (A549) cell line comparing the siSPAG9-treated cancer cells with non-treated cancer cells.

Figure 4 indicates the Western blot analysis of the human lung cancer (A549) cell lines in the presence or absence of siSPAG9.

Figure 5 is a bar chart comparing the percentage of live or viable cells among siSPAG9-treated and non-treated cells.

Figure 6 represents the indirect immunofluorescence analysis of human lung cancer (A549) cells in the presence or absence of siSPAG9 formulation.

Example 1: Determination of endogenous expression of SEQ ID No.17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 in cancer cell lines

A-549 (human lung cancer cells) were grown in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco BRL), 50 units/ml penicillin, and 50 µg/ml streptomycin. The cells were maintained in a humidified 37°C incubator with 5% CO_2. Cancer cells were examined for the expression of protein encoded by SEQ ID 17 or an isoform thereof or expression of polypeptide comprising SEQ ID 18.

The presence protein expressed by SEQ ID 17 or isoform thereof or polypeptide comprising the said SEQ ID 18 in cancer cells was evaluated by indirect immunofluorescence, gel electrophoresis and Western blotting.

Example 2: Indirect Immunofluorescence assay

To determine the presence of protein comprising SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 protein in cancer cells, indirect immunofluorescence assay was performed. Cells were probed with antibodies generated against SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 and subsequently with a secondary labelled antibody (fluorescence conjugated antibody). The presence of fluorescence indicated the endogenous expression of protein comprising SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 protein in cancer cell lines (Figure 1B)

Example 3: Gel electrophoresis and Western blotting

The presence of protein comprising SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 protein may detected by Western blotting procedure wherein cancer cell lysate is run on SDS polyacrylamide gel and transferred onto nitrocellulose matrix.

Briefly, the protein solution was diluted with sample buffer. The samples were then loaded onto polyacrylamide gel. After electrophoresis, proteins were transferred onto

nitrocellulose membrane. Blocked membrane was probed with antibodies generated against SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 and subsequently with a secondary labelled antibody (enzyme conjugated antibody). Finally, membrane was treated with 0.05% DAB.

Western blot analysis of cell lysates from various cancer cell lines demonstrated a strong expression of SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18. Figure 2 which shows a representative photograph of Western Blot analysis of human lung cancer (A549) cell lysate.

Example 4: Inhibition of protein expression of SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18

Preparation of RNAi plasmids:

A general strategy for constructing an RNAi plasmid involved cloning an inverted repeat of nucleotide-sequences from Seq. ID 1-16 of the SPAG9 into conventional expression vector containing U6 promotor. The siSPAG9 for 638 Seq.I D 1 was designed as under:.

638 siSPAG9

AGA TCT CAG TGG ATA TAA A (19 mer) TT so total is 21 mer.

Target sequence 638

Apa I Spacer 9 mer EcoRI

GGG CCC AGA TCT CAG TGG ATA TAA A TTCAAGAGA TTT ATA TCC ACT GAG ATC T TTTTTT GAATTC

CCC GGG TCT AGAGTC ACC TAT ATT T AAGTTCTCT AAA TAT AGG TGA CTC TAGAAAAAAACTTAAG

siRNA 638 complete construct with Apa I site at 5' and EcoRI at 3 'end.

5' GGG CCC AGA TCT CAG TGG ATA TAA A TTCAAGAGA TTT ATA TCC ACT GAG ATC T TTTTTT GAATTC 3'

3' CCC GGG TCT AGA GTC ACC TAT ATT T AA GTTCTCT AAATAT AGG TGA CTC TAGAAAAAAA CTTAAG 5'

Therefore the following primers were designed:

Forward 638 (Oligo 1)

5' GGG CCC AGA TCT CAG TGG ATA TAA A TTCAAGAGA TTT ATA

Reverse 638 (Oligo 2)

GAATTC A AAA AAA GAT CTC AGT GGA TAT AAA TCT CTT GAA TTT ATA

One step PCR was performed and insert was sub-cloned into conventional expression vector containing U6 promotor.

Example 5: siRNA transfection

The siRNA was delivered to various cancerous cell lines and tested for efficacy. The assays were conducted various cancer cell lines of different origin i.e. of ovary, breast, lung, cervix, colon, liver, prostrate, skin, uterus, kidney, urinary bladder, endometrial, bone, pancreas, rectum, pharynx, vulva, placenta, brain, testis, eye, stomach, etc. In all the assays, the siRNA successfully inhibited expression of protein encoded by SEQ ID 17 or an isoform thereof or a polypeptide comprising SEQ ID 18. The siRNAs employed were selected from table 1. A typical example of an assay performed is described below:

Cancer cells were cultured in RPMI (Invitrogen) supplemented with 10% of heat inactivated Fetal calf serum and were grown in 35 mm plates. For siRNA transfection in aqueous medium, the siRNA plasmids were delivered using cellular uptake-enhancing peptide segment or agent. A range of 1 to 12 μg concentration of plasmid DNA was evaluated for inhibiting the expression of SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 and found to be effective in a dose dependent manner.

The reduction in the expression of protein encoded by SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 using siSPAG9 was evaluated by indirect immunofluorescence assay, gel electrophoresis and Western blotting as described above in examples 2 and 3. Further effect on cell viability and apoptosis was also determined in the presence or absence of siSPAG9.

Indirect immunofluorescence analysis of cancer cells revealed a drastic reduction in the expression of protein encoded by SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 to near background levels in the presence of siSPAG9 as shown in Figure 3B, whereas strong fluorescence of SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 (Figure 3A) was observed in non-treated cancer cells.

In Western blot analysis, a drastic knockdown of SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 expression was observed in

siSPAG9 treated cells, whereas the untreated cells revealed no inhibition in the expression of protein encoded by SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18. As shown in Figure 4, lane 1 represents non-treated cancer cells and lane 2 is siSPAG9-treated cancer cells. In lane 1, the cells exhibit expression of protein encoded by SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID No. 18 whereas the lane 2 cells do not show expression of protein encoded by SEQ ID No. 17 or an isoform thereof or a polypeptide comprising the said SEQ ID 18.

Cell viability was determined using the vital dye fluorescein diacetate (FDA). Fluorescein diacetate (FDA) and propidium iodide (PI) were added to a cell sample, which was placed in a hemacytometer observed through a fluorescent filter. The cells that appeared bright green (FDA) were counted and recorded as live cells (Figure 5). The cells were then observed through a rhodamine filter, and cells that appeared bright red (PI) were counted and designated as the number of dead cells. To determine the total cell number, cells were observed under standard light. The percentage of live cells is shown in the bar chart of Figure 5. The non-treated cells were live, whereas viability of siSPAG9 treated cells is reduced to about 5%.

Apoptosis indicator assay may be used to recognize cells dying as a result of apoptosis rather than accidental forms of cell deaths. siSPAG9 treated and non-treated cancer cells were exposed to two fluorescent dyes: fluorescein diacetate (FDA), which stains cells with intact membranes, and propidium iodide (PI), which characterizes cells with compromised membranes.

The presence of apoptotic cells was confirmed by staining with propidium iodide. The induction of apoptosis was not due to any toxic effects intrinsic to the siSPAG9 formulation. This was evident by the absence of apoptotic cells in cultures, wherein no siSPAG9 was introduced. Figure 6A represents live cells stained with FDA and Figure 6B represents dead cells after siSPAG9 formulation treatment.

Example 6: Agarose overlay and formulation:

siRNA may be delivered by gel based formulations. Established cultures of cells of tumor origin may be overlaid with an agarose/liposome/siRNA gel formulation without any adverse effects on cell viability or proliferation.

Briefly, Low melting point agarose was used for agarose overlay method of siRNA delivery into cells. To prepare cells for agarose overlay, they were subcultured into either 96-well or 24-well plates and allowed to establish normally in culture for 24 hours. The medium was then removed, and the cells were washed once with optimal medium and overlaid with molten agarose. The agarose was allowed to set at ambient temperature before incubation at 37°C. Finally, normal antibiotic-free cell culture medium was added to each well, and the cells were cultured up to 72 hours. For preparation of agarose/liposome/siRNA formulation, agarose was diluted with preprepared siRNA-liposomes prepared for routine transfection. After careful mixing, the formulation was applied to the cells as for agarose alone. A formulation of agarose/liposomes (without siRNA) was also tested and found to be equivalent to agarose gel alone in terms of lack of effect on cell growth and viability.

Thus, the applicant demonstrates successful topical gel-based delivery of inducers of RNAi to human epithelial cancer cells. Topical induction of RNAi opens an important new therapeutic approach for treatment of human diseases, including cervical cancer and other accessible disorders.

WE CLAIM:

1. A small interfering ribonucleic acid (siRNA) for inhibiting the expression of a protein encoded by SEQ ID 17 or an isoforn thereof in a cell and polypeptide comprising SEQ ID 18, wherein the siRNA comprises at least 2 sequences that are complementary to each other and wherein a sense strand comprises a first sequence and an anti-sense strand comprises a second sequence comprising a region of complementarity, which is substantially complementary to at least a part of an mRNA encoding a nucleotide sequence from SEQ ID 17, wherein the said siRNA upon contact with a cell expressing protein encoded by SEQ ID 17 or an isoform thereof or expression of polypeptide comprising SEQ ID 18 in a cell

2. A cell comprising the siRNA of claim 1.

3. A vector comprising the siRNA of claim 1.

4. A pharmaceutical composition inhibiting expression of protein encoded by SEQ ID 17 or an isoform thereof in a cell, comprising the siRNA of claim 1 together with a cellular uptake enhancing peptide segment or agent.

5. A method of inhibiting cellular growth/proliferation comprising the step of delivering to the cells the composition of claim 4.

6. A method of causing cell death which comprises the step of delivering to the cell a composition comprising small interfering ribonucleic acid (siRNA) for inhibiting the expression of a protein encoded by SEQ ID 17 or an isoform thereof or expression of polypeptide comprising SEQ ID 18 in a cell, wherein the siRNA comprises at least 2 sequences that are complementary to each other and wherein a sense strand comprises a first sequence and an anti-sense strand comprises a second sequence comprising a region of complementarity, which is substantially complementary to at least a part of an mRNA encoding a polynucleotide sequence from SEQ ID 17, together with an appropriate cellular uptake-enhancing peptide segment or agent.

7. Use of siRNA of claim 1 for preparation of a medicament useful in inhibiting cellular growth/proliferation of cancerous tissues.

8. Use as claimed in claim 7, wherein the cancerous tissues are selected from testis, kidney, uterus, nervous tissue, eye, pituitary, colon, skin, lung, placenta, stomach, urinary bladder, leukopheresis, breast, vulva, pharynx, placenta, bone, prostate and liver.

9. A small interfering ribonucleic acid (siRNA), a cell, a vector, a pharmaceutical composition, a method and use, substantially as hereindescribed with reference to the accompanying examples and drawings.