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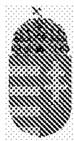

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(54) **Vegyületek és oligonukleotidok rák, és rák-megelőző állapot diagnosztizálására és kezelésére**

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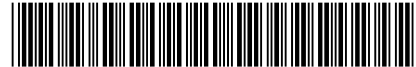

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(54) **Markers for pre-cancer and cancer cells and the method to interfere with cell proliferation therein**

Marker für präkanzerose und kanzerose Zellen sowie Verfahren zur Störung der Zellproliferation darin

Marquers pour cellules précancéreuses et cancéreuses et méthode permettant de perturber la prolifération cellulaire

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The file contains technical information submitted after the application was filed and not included in this specification

Description

FIELD OF THE INVENTION

5 **[0001]** The invention relates to cancer therapy, cancer diagnosis and research reagents in connection with a novel family of human mitochondrial RNAs referred to as human mitochondrial chimeric RNAs. In particular, this invention relates to oligonucleotides targeted to the human mitochondrial chimeric RNAs. The oligonucleotides of the invention hybridize to the chimeric RNAs inducing cancer cell death. The compositions provided in the invention are useful as a new cancer therapy. In addition, the oligonucleotides can be used for diagnosis of cancer and pre-cancer cells based
10 on the differential expression of the mitochondrial chimeric RNAs in resting and proliferating normal cell, pre-cancer and cancer cells.

BACKGROUND OF THE INVENTION

15 **[0002]** Mitochondria are subcellular organelles that manufacture the essential molecule adenosine triphosphate (ATP) by oxidative phosphorylation. The human mitochondrial DNA (mtDNA) of 16,654 base pairs encodes two ribosomal RNAs, 22 transfer RNAs (tRNAs) and 13 open reading frames (ORF) that encode a similar number of polypeptides (Clayton, Hum Reprod., Suppl. 2:11-17, 2000; Taanman, Biochim. Biophys. Acta, 1410:103-123, 1999). On the basis of the content of G+T base composition, the two strands of the mtDNA differ in buoyant density and can be separated
20 in denaturating cesium chloride gradients. The heavy strand or H-strand encodes the two ribosomal RNAs (12S and 16S), 14 tRNAs and 12 polypeptides corresponding to ND 1, ND 2, ND 3, ND4L, ND4, ND 5, COX I, COX II, COX III, ATP6, ATP8 and Cyt b. The light strand or L-strand codes for 8 tRNAs and the subunit of the complex NAD dehydrogenase ND6 (Clayton, Hum Reprod., Suppl. 2:11-17, 2000; Taanman, Biochim. Biophys. Acta, 1410:103-123, 1999).

[0003] A large proportion of the mtDNA contains a short three-stranded structure called the displacement loop or D-
25 loop. This region, that in humans is 1,006 base pairs, is flanked by the genes for tRNA of phenylalanine ($tRNA^{Phe}$) and the tRNA of proline ($tRNA^{Pro}$) and contains a short nucleic acid strand complementary to the L-strand and displacing the H-strand (Clayton, Hum Reprod., Suppl. 2:11 -17, 2000; Taanman, Biochim. Biophys. Acta, 1410:103-123, 1999). This region has evolved as the major control site for mtDNA expression and contains the leading-strand or H-strand origin of replication and the major promoters for transcription of the H- (HSP) and L-strand (LSP). Despite the close
30 proximity of the HSP and LSP (about 150 bp), these regulatory elements are functionally independent *in vitro* (Shuey and Attardi, J. Biol. Chem., 260:1952-1958, 1985; Taanman, Biochim. Biophys. Acta, 1410:103-123, 1999) as well as *in vivo,* utilizing model patients with mitochondrial diseases (Chinnery and Turnbull, Mol. Med. Today, 6:425432, 2000).

[0004] Both strands are transcribed as polycistronic RNAs which are then processed to release the individual mRNAs, tRNAs and the rRNAs (Taanman, Biochim. Biophys. Acta, 1410:103-123, 1999). In humans, the mitochondrial RNA
35 polymerase is a protein of 1,230 amino acids with significant homology with the sequence of yeast mitochondrial RNA polymerase and with the RNA polymerases of several bacteriophages (Tiranti et al., Hum Mol Genet., 6:615-625, 1997). In addition, a family of transcription factors has been characterized such as the mitochondrial transcription factor A or TFAM which is essential for mammalian mtDNA transcription and is a member of the high mobility group (HMG)-box family of DNA-binding proteins (Paris) and Clayton, Science, 252:965-969, 1991). Recently, two independent reports
40 described the characteristics of new transcription factors, TFB1M and TFB2M, in human and mouse (McCulloch et al., Mol. Cell Biol., 22,1116-1125, 2002; Falkenberg et al., Nat. Genet., 31:289-294, 2002; Rantanen et al., Mamm. Genome, 14:1-6, 2003). In spite of the considerable progress achieved on the *cis*-and *trans*-acting elements involved in mtDNA transcription, the functional details are not fully understood.

[0005] Villegas et al., DNA and Cell Biology, 2000, Vol 19, No 9, pp579-588 describes a chimeric mitochondrial RNA
45 localized in the nucleus of mouse sperm. The chimeric mitochondrial RNA comprises an inverted repeat joined to the 5' end of the 16S mitochondrial RNA. Villegas et al., Nucleic Acids Research, 2002, Vol 30, No 9, pp1895-1901 further characterizes this chimeric RNA, and discusses a putative RNA editing from U to C in a mouse mitochondrial transcript. Villegas et al., Molecular Human Reproduction, 2002, Vol 8, No 11, pp977-983 describe the localization of the 16S mitochondrial rRNA in the nucleus of mammalian spermatogenic cells.

50

Mitochondria and Apoptosis

[0006] Mitochondria play a central role in apoptosis, a fundamental biological process by which cells die in a well-controlled or programmed manner. This cell suicide program is essential during development and for adult homeostasis
55 of all metazoan animals. Apoptosis is activated to eradicate superfluous, damaged, mutated and aged cells (Meter et al., Nature, 407:796-801, 2000). Disregulation of apoptosis is implicated in the appearance of several pathologies. Thus, abnormal inhibition of apoptosis is a hallmark of neoplasia, whereas massive apoptosis has been linked with acute diseases such as stroke, septic shock and neurodegenerative disorders. At present the process of apoptosis is described

as two major pathways known as the extrinsic and the intrinsic pathways (Zornig et al., Biochim. Biophys. Acta, 1551:F1-F37, 2001). The extrinsic pathway is a process that is initiated at the cell membrane by the binding of different ligands to the death receptors (Krammer, Mature, 407:789-795, 2000; Zornig et al., Biochim. Biophys. Acta, 1551:F1-f37, 2001).

[0007] Caspases are responsible for the proteolytic cascade in apoptosis. Caspases are synthesized as inactive precursor proteins that undergo proteolytic maturation or processing upon apoptosis induction (Zornig et al., Biochim. Biophys. Acta, 1551:F1 - F37, 2001). However, more recently several experimental studies indicate that lysosomal proteases constitute an alternative pathway of proteolysis after apoptotic insults (Guicciardi et al., Oncogene, 23:2881-2890, 2004). On the other hand, anti-apoptotic proteins homologous to the human oncoprotein Bcl-2 have been described. This protein belongs to a family of proteins that are either anti-apoptotic (Bcl-2, Bcl-XL, Bcl-w) or pro-apoptotic (Bax, Bak, Bim, Bid, etc.) (Zorning et al., Biochim. Biophys. Acta, 1551:F1-F37, 2001).

[0008] Mitochondria are particularly affected early during the apoptotic process and at the present time they are recognized as the central coordinators of cell death (Boya et al., Biochem. Biophys. Res. Commun., 304:575-581, 2003; Ferri and Kroemer, Nature Cell Biol., 3:E255-E263, 2001; Zornig et al., Biochim. Biophys. Acta, 1551:F1-F37, 2001). Several pro-apoptotic signal and damage pathways converge on mitochondria to induce mitochondrial membrane permeabilization, a phenomenon that is under the control of Bcl-2 proteins (Boya et al., Biochem. Biophys. Res. Commun., 304:575-581, 2003; Zornig et al., Biochim. Biophys. Acta, 1551:F1-F37, 2001). After cells receive apoptotic insults, the trans-membrane potential ($\Delta\psi_m$) dissipates resulting in the complete permeabilization of the outer mitochondrial membrane and the consequent leakage of toxic mitochondrial intermembrane proteins. The first example of the leakage of a mitochondrial protein was the liberation of cytochrome c (Liu et al., Apoptosis, 6:453-462, 2001). When cytochrome c is present in the cytosol, it drives the assembly of the caspase activating complex termed the apoptosome. Cytochrome c binds to Apaf-1 (apoptotic protease activation factor-1) facilitating the binding of dATP/ATP to the complex and the oligomerization of Apaf-1 (Adrain et al., J. Biol. Chem., 274:20855-20860, 1999; Benedict et al., J. Biol. Chem., 275:8461-8468, 2000). Oligomerization of Apaf allows the recruitment of pro-caspase-9 which catalyzes the proteolytic activation of the precursor and generation of active caspase-9 (Adrain et al., J. Biol. Chem., 274:20855-20860, 1999; Benedict et al., J. Biol. Chem., 275:8461-8468, 2000).

[0009] A family of cytosolic inhibitor of apoptosis proteins has been described and is known as XIAP, c-IAP1 and c-IAP2. These proteins bind to and inhibit processed caspase-3 and caspase-9 and consequently stop the cascade of degradation. However, the cell also contains countermine mechanisms to bypass this anti-apoptotic pathway. In cells undergoing apoptosis, caspases are liberated of this inhibitory effect by binding to IAPs of a protein known as Smac (Second Mitochondrial Activator of Caspases) or DIABLO (Direct IAP Binding protein with Low p1) (Verhagen et al., Apoptosis, 7:163-166, 2002). By binding to IAPs, Smac/DIABLO displace active caspases from IAPs and thus promote cell death. Another protein, known as HtrA2, is released from the mitochondria into the cytosol after apoptotic insult where the protein binds to IAPs in a similar fashion as does Smac/DIABLO and thereby facilitating caspases activation (Verhagen et al., Apoptosis, 7:163-166, 2002; Martins et al., 2001; Suzuki et al., Mol. Cell, 8:613- 621, 2001; Hedge et al., Apoptosis, 7:123-132, 2002).

[0010] The apoptosis inducing factor (AIF) is another component of the apoptotic cascade. After induction of apoptosis, AIF translocates to the cytosol and to the nucleus. In the nucleus, AIF induces peripheral chromatin condensation and DNA fragmentation. AIF also induces several hallmarks of apoptosis like $\Delta\psi_m$ dissipation and phosphatidylserine exposure (Zornig et al., Biochim. Biophys. Acta, 1551:F1- F37, 2001). A factor that seems to regulate the apoptotic activity of AIF is the heat schock protein 70 (Ravagnan et al., Nature Cell Biol., 3:839-843, 2001). Another mitochondrial factor that exits the mitochondria and translocates into the nucleus like AIF is endonuclease G or Endo G. In the nucleus, Endo G generates DNA fragmentation even in the presence of caspase inhibitors (Li et al., Mature, 412:95- 99, 2001). Endo G may act in similar fashion as CAD (caspase-activated DNAse), a nuclease whose activation critically relies on caspases (Samejima et al., J. Biol. Chem., 276:45427-45432, 2001).

Cancer and Pre-cancer

[0011] Cancer is a cellular malignancy whose unique trait, loss of normal control of cell cycle, results in unregulated growth, lack of differentiation, and ability to invade other tissues and metastasize. Carcinogenesis is the process by which a normal cell is transformed in a malignant cell. Carcinogenesis is a multiple step process beginning with the genetic event of initiation followed by selective expansion of altered cells during promotion to form early adenomas. In the absence of continuous promotion, the adenoma regresses and disappears. With a second genetic event, a small number of promoted adenomas progress to form late adenomas some of which may then undergo malignant conversion (McKinnell et al., "The Biology Basis of Cancer", Ch. 3, 1998).

[0012] The etiology of cancer is complex and includes alteration of the cell cycle regulation, chromosomal abnormalities and chromosome breakage. Infectious agents such several types of oncogenic viruses, chemicals, radiation (ultraviolet or ionizing radiation) and immunological disorders are thought to be the major causes of carcinogenesis (McKinnell et al., "The Biological Basis of Cancer", Ch. 3, 1998).

[0013] It has been proposed for a long time that cancer is also related to mitochondrial dysfunction. One of these theories suggests that mitochondrial mutation might be the primary cause of cell transformation and cancer (Warburg, 1956; Carew and Huang, Mol. Cancer, 1:1-12, 2002). Alterations of the mitochondrial DNA (mtDNA) were reported in hematologic malignancies (Clayton and Vinograd, Mature, 216:652-657, 1967) and in breast cancer (Tan et al., 2002; Parrella et al., 2001). Mutations of several regions of the mtDNA and deletions have been also identified in patients with colorectal cancer, prostate cancer, ovarian cancer, gastric cancer, pancreatic cancer, hepatocellular carcinoma, esophageal cancer, kidney cancer, thyroid cancer and brain tumors (reviewed by Carew and Huang, Mol. Cancer, 1:1-12, 2002). In general, there appear to be two major features of mtDNA alterations in cancer irrespective of tumor type. The majority of the mutations are base transitions from T to C and G to A. Second, while there is diversity in the particular genes in which mutations occur, the D-loop seems to be the most frequent somatic mutated region of the mtDNA in most tumor types.

[0014] Pre-cancer cells are defined here as a transformed cell which can evolve or differentiate into a malignant cell. Some examples are cells transformed by DNA or RNA oncoviruses.

[0015] The present disclosure is related to a novel family of mitochondrial RNAs and the use herein of these RNAs as targets for diagnostics and cancer therapy. The present disclosure provides compositions and that are useful to differentiate normal cells from tumor cells, or from pre-malignant cells or cells transformed with oncogenic viruses. In particular, as elaborated below, the present disclosure provides compositions for diagnostic assays to differentiate normal cells from pre-cancer and cancer cells. In another embodiment of the disclosure, compositions are provided to induce massive and selective tumor cell death. Therefore, the present disclosure provides compositions which may be used in cancer and pre-cancer therapy as well as for research.

SUMMARY OF THE INVENTION

[0016] In accordance with the present invention, there is provided a pharmaceutical composition, wherein the pharmaceutical composition comprises one or more antisense oligonucleotides of 10-50 nucleobases in length which are complementary to a human mitochondrial chimeric RNA molecule comprising a sense or antisense 16S mitochondrial RNA covalently linked at its 5' end to the 3' end of a polynucleotide with an inverted repeat sequence, to be able to hybridize with that human mitochondrial chimeric RNA molecule to form a stable duplex, further wherein one or more of the oligonucleotides comprise at least one alternate internucleoside linkage.

Sense mitochondrial chimeric RNAs

[0017] In one aspect of this disclosure compositions are provided to detect a sense mitochondrial chimeric RNA comprised of an inverted repeat of 815 nucleotides joined covalently to the 5' end of the 16S mitochondrial ribosomal RNA (SEQ ID NO 1). The inverted repeat corresponds to a fragment of 815 nucleotides of the RNA transcribed from the L-strand of the 16S gene of the mtDNA. Thus, the synthesis of this novel RNA requires the transcription of the L-strand and the H-strand of the 16S gene of the mtDNA. Since transcription of both strands of the mtDNA is regulated by different promoters, we refer to this novel RNA in the present invention as the mitochondrial chimeric RNA (SEQ ID NO 1). In addition, since the inverted repeat of 815 nucleotides is joined to the "sense" 16S RNA (transcribed from the H-strand) we refer to this novel RNA as the "sense mitochondrial chimeric RNA". This disclosure provides compositions to detect the expression of the sense mitochondrial chimeric RNA in cultured cells, in cell samples, and in tissue sections. The detection can be carried out by in situ hybridization, synthesis of the corresponding cDNA and amplification by PCR, transcription mediated amplification (TMA) (Comanor et al., J. Clin Virol., 28:14-26, 2003) or Northern blot, or other methods obvious to one skilled in the art.

[0018] *In situ* hybridization assays revealed that the sense mitochondrial chimeric RNA is expressed in normal proliferating cells, in tumor cells in culture as well as in tumor cells present in human biopsies of different tumor types. The sense mitochondrial chimeric RNA is not expressed in normal resting cells. In yet another embodiment of the disclosure, compositions are provided to detect a second novel sense mitochondrial chimeric RNA in cells transformed with papilloma virus 16 or 18 (Heusen, Biochim. Biophys. Acta, 1288:F55-F78, 1996). In these transformed cells, a new sense mitochondrial chimeric RNA comprising of an inverted repeat of 754 nucleotides joined covalently to the 5' end of the 16S mitochondrial RNA is expressed (SEQ ID NO 2). This RNA is not present in normal proliferating cells or in tumor cells. The compositions also demonstrated that a third sense mitochondrial chimeric RNA, comprising an inverted repeat of 694 nucleotides joined covalently to the 5' end of the 16S mitochondrial RNA (SEQ ID NO 3), is present in cells transformed with HTLV1.

Antisense mitochondrial chimeric RNA

[0019] This disclosure also provides compositions that revealed that normal proliferating cells over express antisense

mitochondrial chimeric RNAs corresponding to SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6. These transcripts contain inverted repeats of variable length (transcribed from the H-strand) joined to the 5' end of the antisense 16S mitochondrial ribosomal RNA (transcribed from the L-strand), hence the name of antisense mitochondrial chimeric RNA. The expression of the antisense mitochondrial chimeric RNA is down regulated in tumor cell lines in culture as well as in tumor cells present in human biopsies of different types of tumors as well as in transformed or pre-cancer cells. Accordingly, the present disclosure provides compositions to detect the expression of the sense and the antisense mitochondrial chimeric RNAs, distinguishing normal proliferating cells from cancer and pre-cancer cells and therefore provides a novel marker for malignant cells and cancer.

Cancer therapy

[0020] In another aspect of this invention, compositions are provided to interfere with the sense and antisense mitochondrial chimeric RNAs. One preferred embodiment is to interfere with the antisense mitochondrial chimeric RNA in tumor cells which contains a low copy number of this transcript. The interference is carried out with oligonucleotides or oligonucleotide analogs, whose sequences are complementary to the sequences of the antisense mitochondrial chimeric RNA (SEQ ID NO 4 and/or SEQ ID NO 5 and/or SEQ ID NO 6). Treatment of tumor cells of different types with one or more of these complementary oligonucleotides induces cell death or apoptosis. The treatment of pre-cancer or cancer is carried out with compounds or oligonucleotides 10 to 50 nucleobases in length which are sufficiently complementary to one human mitochondrial chimeric RNA molecule comprising a sense 16S mitochondrial ribosomal RNA covalently linked at its 5' end to the 3' end of a polynucleotide with an inverted repeat sequence to be able to hybridize with that human mitochondrial chimeric RNA molecule to form a stable duplex. The oligonucleotides are preferably compounds of 15 to 50 nucleotides where at least 15 nucleobases are complementary to SEQ ID NO 4 and/or SEQ ID NO 5 and/or SEQ ID NO 6. Examples of these complementary oligonucleotides are shown in SEQ ID NOS 98 to 196. The induction of apoptosis is selective since treatment of human lymphocytes (normal resting cell) or human lymphocytes stimulated with phytohaemagglutinin (normal proliferating cells) do not undergo apoptosis after treatment with oligonucleotides complementary to the sequences of the antisense mitochondrial chimeric RNA under the same conditions. If the tumor cells are treated with oligonucleotides targeted or complementary to the sense mitochondrial chimeric RNA (SEQ ID NO 1 and/or SEQ ID NO 2 and/or SEQ ID NO 3) a diminished induction of cell death or apoptosis is obtained.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021]

FIGS. 1A, B and C. Line drawings showing the structure of sense mitochondrial chimeric RNAs corresponding to SEQ ID NO 1, SEQ ID NO 2 and SEQ ID NO 3. The arrows indicate the relative position of the primers used to amplify the RNA by pieces. The arrows below the lines are reverse primers, and the arrows on top of the lines are forward primers. Primer 1 is positioned close to the 5' end of the 16S mitochondrial RNA. The black lines correspond to the sense 16S mitochondrial RNA, while the dotted lines correspond to the antisense 16S mitochondrial RNA.

Fig. 2. Agarose gel electrophoresis to show the amplification product obtained between primer 1 and primer 3 indicated in FIG. 1A. The amplification was carried out by RT-PCR using as a template a tumor cell (SiHa), human keratinocytes transformed with HPV 16 (HFK698) or B-lymphocytes transformed with HTLV-1. With RNA from SiHa cells only one single amplicon of 210 bp was obtained and corresponds to a segment of SEQ ID NO 1. In total RNA of keratinocytes transformed with HPV 16, besides the amplicon of 210 bp, a second amplicon of 150 bp was obtained and corresponds to a segment of SEQ ID NO 2. With RNA from cells transformed with HTLV-1, besides the amplicons of 210 bp and 150 bp, a third amplicon was obtained and corresponds to a segment of SEQ ID NO 3.

FIG. 3. Line drawings showing the structure of the antisense mitochondrial chimeric RNAs corresponding to SEQ ID NO 4, SEQ ID NO 5 and SEQ ID NO 6. The arrows represent the primer used for amplification, and primer 1 is positioned close to the 5' end of the antisense 16S mitochondrial RNA. The strategy to obtain the sequence of these transcripts is similar to that described in Fig. 1.

FIGS. 4A and 4B. *In situ* hybridization assays carried out with several tumor cell lines in culture. The cells were hybridized with oligonucleotide probes complementary to the sense mitochondrial chimeric RNA, and labeled with digoxigenin (left panels). In addition, the cells were also hybridized in parallel with oligonucleotide probes complementary to the antisense mitochondrial chimeric RNA labeled with digoxigenin (right panels). The cells lines are identified at the left.

FIG. 5A. *In situ* hybridization of several sections of human biopsies corresponding to different tumor types. The tumor sections were hybridized with oligonucleotide probes complementary to the sense mitochondrial chimeric RNA, and labeled with digoxigenin (left panels). In addition, parallel tumor sections were hybridized with oligonucleotide probes complementary to the antisense mitochondrial chimeric RNA labeled with digoxigenin (right panels).

Fig. 5B shows *in situ* hybridization of different human tumors carried out with oligonucleotide probes complementary to the sense mitochondrial chimeric RNA labeled with digoxigenin.

FIG. 6 shows *in situ* hybridization of normal proliferating cells. The samples were hybridized with probes targeted to the sense or the antisense mitochondrial chimeric RNA and labeled with digoxigenin. A strong hybridization signal was obtained with both probes, one complementary to the sense mitochondrial chimeric RNA (left panels) as well as to the antisense mitochondrial chimeric RNA (right panels). The tissues or cells are identified at the left.

FIG. 7. Immunocytochemistry and *in situ* hybridization to show expression changes in human lymphocytes stimulated to proliferate with the mitogen PHA. After 48 hours of stimulation with PHA, the lymphocytes express the antigens Ki-67 and PCNA (right panels). These antigens are not expressed in the control or resting lymphocytes (left panels). The *in situ* hybridization was carried out with oligonucleotide probes targeted to the sense and the antisense mitochondrial chimeric RNA and labeled with digoxigenin. The stimulated lymphocytes over express the sense as well as the antisense mitochondrial chimeric RNA (right panels).

Fig. 8. *In situ* hybridization of tumor cells showing localization of the sense mitochondrial chimeric RNA in the nucleolus. The cells or tumor sections are indicated at the left.

FIG. 9. Fluorescent microscopy to reveal changes occurring in tumoral HL- 60 cells treated with oligonucleotide probes targeted to the antisense mitochondrial chimeric RNA. A, B. C and D show staining with a compound (VAD-fmok) that binds with high affinity to activated caspases. This compound is labeled with fluorescein. The oligonucleotide probes targeted to the antisense mitochondrial chimeric RNA induce activation of caspases in similar manner than the drug staurosporine (compare B and C). Activated caspases are not detected in control untreated cells (A) or in cells treated with oligonucleotide probes targeted to the 12S mitochondrial RNA (D), as control. E and F show staining of HL-60 cells with DAPI. The control cells (untreated) show homogeneous staining of the nucleus (E), while cells treated with the oligonucleotide probes targeted to the antisense mitochondrial chimeric RNA show massive fragmentation of the nucleus (F).

FIG. 10. Percent of apoptotic cells after different treatment conditions of resting and proliferating lymphocytes. Apoptosis was measured in resting lymphocytes or PHA-stimulated lymphocytes by DAPI staining. The bars 1 and 2 correspond to untreated cells. A low spontaneous apoptosis of control (1) or PHA-stimulated lymphocytes (2) was observed. A similar low level of apoptosis was observed in resting lymphocytes (3) or PHA-stimulated lymphocytes (4) treated with 15 uM oligonucleotide probes targeted to the antisense mitochondrial chimeric RNA for 15 hours, showing that apoptosis is not induced in normal cells. As a control, resting lymphocytes and PHA-stimulated lymphocyte were treated with staurosporine. Under these conditions, around 90% of resting lymphocytes (5) or PHA-stimulated lymphocytes (6) undergo apoptosis.

DETAILED DESCRIPTION OF THE INVENTION

The human mitochondrial chimeric RNA family

[0022] The present invention is based on the surprising discovery that human cells express a family of novel mitochondrial RNAs, referred to as the human mitochondrial chimeric RNAs.

[0023] One of these transcripts contains a long inverted repeat of 815 nucleotides covalently joined to the 5' end of the mitochondrial 16S ribosomal RNA, named sense mitochondrial chimeric RNA. The long inverted repeat is fully complementary to the 16S ribosomal RNA from positions 51 to 866, forming a long double stranded stem and a loop of 50 nucleotides. As shown in Fig. 1A, the stem of 815 base pairs represents a significant problem for any reverse transcriptase to synthesize the corresponding cDNA. Therefore a new strategy was used to amplify this RNA by RT-PCR which is illustrated in Fig. 1A. After obtaining the sequence of each overlapping fragment, they were assembled as contigs to obtain the complete sequence of the sense mitochondrial chimeric RNA shown in SEQ ID NO 1 (Fig. 1A).

[0024] Another aspect of this disclosure is the discovery of other novel sense mitochondrial chimeric RNAs which are expressed in cells transformed with the oncogenic human papilloma virus 16 or 18. Human foreskin keratinocytes (HFK) where infected with HPV 16 or 18 (Heusen, Biochim. Biophys. Acta, 1 288:F55-F78, 1996). The infection induces

transformation or immortalization of the HFK. However, these cells are not tumorigenic such as the related SiHa cells (infected with HPV 16) or HeLa cells (infected with HPV 18). These cells express the sense mitochondrial chimeric RNA (SEQ ID NO 1) similar to SiHa and HeLa cells. However, the transformed cells also express another second sense mitochondrial chimeric RNA which contains an inverted repeat of 754 nucleotides joined to the 16S ribosomal RNA (Fig. 1B) (SEQ ID NO 2). This new sense mitochondrial chimeric RNA is down regulated or is not expressed in normal human cells (HFK) or in tumorigenic cells (SiHa or HeLa cells).

[0025] In another embodiment of this discovery we determined the expression of a third sense mitochondrial chimeric RNA in cells transformed with HTLV-1 (Kobayashi et al., EMBO J., 3:1339-1343, 1984). MT-2 cells infected with HTLV-1 express the sense mitochondrial chimeric RNA (SEQ ID NO 1) and the sense mitochondrial chimeric RNA expressed in cells transformed with HPV 16 or 18 (SEQ ID NO 2). Besides these transcripts, the cell infected with HTLV-1 expresses a third sense mitochondrial chimeric RNA containing an inverted repeat of 694 nucleotides joined to the 5' end of the 16S ribosomal RNA. This novel RNA (Fig. 1C) (SEQ ID NO 3) is not expressed in normal proliferating cells, in tumor cells or in HFK transformed with HPV 16 or 18.

[0026] Normal proliferating cells such as human foreskin keratinocytes (HFK) as described in the previous section also over express the sense mitochondrial chimeric RNA (Fig. 6) (SEQ ID NO 1). Human lymphocytes stimulated with mitogens such as phytohaemagglutinin (PHA) enter into the S phase of the cell cycle and begin the synthesis of DNA (Yu et al., J. Biol. Chem., 266:7588-7595, 1991). As proliferating cells, the lymphocytes also express antigens related to proliferation such as Ki-67 and proliferating cell nuclear antigen or PCNA (Bantis et al., Cytopathology, 15:25- 31, 2004). The stimulated lymphocytes also over express the sense mitochondrial chimeric RNA (SEQ ID NO 1). Other proliferating cells such as lymphocytes in the germinal center of the spleen, spermatogonia, and embryonic cells also over express the sense mitochondrial chimeric RNA (SEQ ID NO 1) (Fig. 4). In contrast, non-proliferating cells such as non-stimulated lymphocytes, or muscle cells do not express the sense mitochondrial chimeric RNA (Fig. 7).

[0027] In another embodiment of the disclosure, compositions useful in methods to differentiate normal proliferating cells from a tumor cell are provided. As described before, tumor and normal proliferating cells over express the sense mitochondrial chimeric RNA described in SEQ ID No 1. In addition, in specific situations of infection with HPV and HTLV-1, additional chimeric RNA are found (SEQ ID NO 2 and SEQ ID NO 3). However, the present invention is also based on the surprising discovery that normal proliferating cells also over express an antisense mitochondrial chimeric RNA. The expression of the antisense mitochondrial chimeric RNA was confirmed in human lymphocytes stimulated with PHA (Fig. 7), in normal HFK and in other normal proliferating cells (Fig. 6). Another surprising discovery of the present invention is that different to normal proliferating cells, tumor cells do not express the antisense mitochondrial chimeric RNA or markedly down regulated the production (compare Fig. 4 with Fig. 6 and Fig. 7).

[0028] Using the same strategy to amplify by RT-PCR the chimeric RNA based in overlapping fragments described earlier, the structure of the antisense mitochondrial chimeric RNA was determined (Fig. 3). The sequencing and assembling in contigs reveals a complex family of antisense mitochondrial chimeric RNAs containing inverted repeats of different lengths joined to the 5' end of the antisense 16S mitochondrial ribosomal RNA (Figs. 3A, B and C) (SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6). The sequence also reveals the formation of double stranded structures or stems in these RNA and the formation of loops with 17, 96 and 451 nucleotides, respectively (Fig. 3A, B and C, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6). In another embodiment of the disclosure, compositions are provided to follow the oncogenic transformation of cells by an oncogenic virus. HeLa cells (infected with HPV 18) or SiHa cells (infected with HPV 16) over express the sense mitochondrial chimeric RNA but down regulate the expression of the antisense mitochondrial chimeric RNAs. On the other hand, HFK as normal proliferating cells, over express both the sense as well as the antisense mitochondrial chimeric RNAs. After transformation of HFK with HPV 16 or HPV 18, the cells acquire the tumor phenotype: they over express the sense mitochondrial chimeric RNA and down regulate the expression of the antisense mitochondrial chimeric RNA. The over expression of the sense mitochondrial chimeric RNA and down regulation of the antisense mitochondrial chimeric RNA can be determined by *in situ* hybridization, amplification of the RNA by RT-PCR or by using other methods to determine a RNA by ways well known to the person skilled in the art. These compositions can be used also to determine the change in the expression of the chimeric RNA family in cells transformed with other oncogenic virus or by compounds that induce transformations or carcinogenesis (McKinnell et al., "The biological basis of Cancer", Cambridge University Press 1998).

Cancer and pre-cancer diagnostics

[0029] According to the present disclosure compositions are provided to detect in a biological sample the presence of the sense mitochondrial chimeric RNAs and the antisense mitochondrial chimeric RNAs. In one preferred embodiment, the detection is carried out by *in situ* hybridization. The detection of the sense mitochondrial chimeric RNA and the antisense mitochondrial chimeric RNAs in the cells of the biological sample indicates that the cells are normal proliferating cells. In another embodiment, the result of the *in situ* hybridization with tumor cells will show expression of the sense mitochondrial chimeric RNA and down regulation or absence of the antisense mitochondrial chimeric RNA. If the biological

sample contains non-proliferating normal cells the *in situ* hybridization will show that neither the sense mitochondrial chimeric RNA nor the antisense mitochondrial chimeric RNA are expressed.

[0030] Biological samples are understood as normal cells (resting or proliferating cells) in culture or in blood smears or bone marrow smears, tumor cells in culture and normal cells transformed with oncogenic virus. Additionally, biological samples comprise cells obtained from the urine or the bladder washing from patients suspecting of having bladder or kidney cancer, or cells from saliva in patients suspecting of having head and neck cancers, or cells from bronchoalveolar gavage from patients suspecting of having lung cancer. Also, biological samples comprise cell smears from the blood of patients suspecting of having leukemia or cell smears from blood or lymph or lymph nodes of patients suspecting of having metastasis.

[0031] The biological samples according to the disclosure include the use of rapidly frozen tissue or cells samples for histopathological analysis, art well known by artisans in the field. Alternatively, the biological sample can be biopsies of sections fixed by using chemical treatment that can be accomplished by the wide variety of fixation protocols known in the art (Frederick et al, Current Protocols In Molecular Biology, Volume 2, Unit 14, Frederick M. Ausubul et al. edS., 1995; Celis, Cell Biology, A Laboratory Handbook, Julio E. Celis, ea., 1994). The biological samples can also be non-fixed biological materials that are not been chemically modified or treated with formalin or other fixative well known in the art.

[0032] Alternatively, the *in situ* hybridization can be carried out by using biological samples embedded in materials such as paraffin or other embedding polymers. The blocks obtained after embedding can be sectioned with a microtome in section of about 4 to about 10 um of thickness. The section can then be applied to glass or plastic slides coated with an adhesive substance known in the art such as polylysine or mussel adhesive protein (Burzio et al., Curr. Opin. Biotechnol., 8:309-312, 1997).

[0033] The *in situ* hybridization of the present disclosure can be carried out in ways well known to persons skilled in the art. For example, a hybridization solution comprising one or more labeled probes targeted to one or more of the sequences of sense mitochondrial chimeric RNA (SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3) or antisense mitochondrial chimeric RNA (SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6) within the cell, is contacted with the cell under hybridization conditions. The hybridization signal is then compared with a predetermined hybridization pattern from normal or control cancer and pre-cancer cells.

[0034] As used herein, the labeled probes to carry out the *in situ* hybridization are RNA, DNA or synthetic nucleic acids that can be prepared by any method known in the art. Synthetic nucleic acids include riboprobes transcribed in vitro or PCR fragments. In a preferred embodiment of this disclosure, synthetic complementary oligonucleotides can be used. The complementary oligonucleotide probes are at least about 10 nucleotides in length, most preferably at least about 14, and most preferably at least 18 nucleotides in length. The skilled artisan understand that the length can extend from 10 nucleotides or more to any length which still allows hybridization to the sense mitochondrial chimeric RNAs or the antisense mitochondrial chimeric RNAs. In a preferred embodiment herein, the length is about 30 nucleotides, more preferably about 25 nucleotides, and most preferably between 10 to 50 nucleotides in length. Longer probing nucleic acids may also be used. The sequence of the probe is at least ninety five percent homologous to the sequences listed in SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5 and SEQ ID NO 6.

[0035] The complementary oligonucleotide probes of the present disclosure will generally contain phosphodiester bonds, although in some cases, oligonucleotide probe analogs are included that may have alternate internucleoside linkages, comprising, but not limited to, phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437-1441, 1991; and U.S. Pat. No. 5, 644,048), peptide nucleic acid or PNA (Egholm, Nature, 365:566568, 1993; and U.S. Pat. No. 6,656,687), phosphoramide (Beaucage, Methods Mol. Biol. 20:33-61, 1993), phosphorodithioate (Capaldi et al., Nucleic Acids Res., 28:E40, 2000). Other complementary oligonucleotide analogs include, but are not limited to, morpholino (Summerton, Biochim. Biophys. Acta, 1489:141-158, 1999), locked oligonucleotides (Wahlestedt et al., Proc. Natl. Acad. Sci. US, 97:5633-5638, 2000), peptidic nucleic acids or PNA (Nielsen *et al.,* 1993; Hyrup and Nielsen, 1996) or 2-o(2-methoxy) ethyl modified 5' and 3' end oligonucleotides (McKay et al., J. Biol. Chem., 274:1715-1722, 1999). The nucleic acid may contain any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc.

[0036] In another embodiment of the disclosure, the nucleic acid or oligonucleotide probes have to be labeled to detect the hybridization with the sense mitochondrial chimeric RNAs or the antisense mitochondrial chimeric RNAs. The probes may be labeled with a detectable marker by any method known in the art. Methods for labeling probes include random priming, end labeling, PCR and nick translation. Enzymatic labeling is conducted in the presence of nucleic acid polymerase, three unlabeled nucleotides, and a fourth nucleotide which is either directly labeled, contains a linker arm for attaching a label, or is attached to a hapten or other molecule to which a labeled binding molecule may bind. Suitable direct labels include radioactive labels such as ^{32}P. ^{3}H. and ^{35}S and non-radioactive labels such as fluorescent markers. Preferred fluorochromes (fluorophores) include 5(6)- carboxyfluorescein, 6-((7-amino-4-methylcoumarin.-3-acetyl)amino)hexanoic acid, 5(and 6)-carboxy-X-rhodamine, Cyanine 2 (Cy2) Dye, Cyanine 3 (Cy3) Dye, Cyanine 3.5 (Cy3.5) Dye, Cyanine 5 (Cy5) Dye, Cyanine 5.5 (Cy5.5) Dye Cyanine 7 (Cy7) Dye, Cyanine 9 (Cy9) Dye (Cyanine dyes 2, 3, 3.5, 5

and 5.5 are available as NHS esters from Amersham, Arlington Heights, Ill.) or the Alexa dyes comprising Alexa 488, Alexa 532, Alexa 556, Alexa 590, etc. (Molecular Probes, Eugene, Oreg.).

[0037] Probes may be indirectly labeled by incorporating a nucleotide covalently linked to a hapten or other molecule. Preferred haptens, but not limited to, include 5(6)-carboxyfluorescein, 2,4-dinitrophenyl, digoxigenin and biotin, and performing the detection of the probe with a labeled antibody directed to that hapten or other molecule. In the case of biotin, detection can be carried out with avidin or streptavidin conjugated to a detectable label. Antibodies, streptavidin and avidin may be conjugated with a fluorescent marker, or with an enzymatic marker such as alkaline phosphatase or horseradish peroxidase to render them detectable. Conjugated streptavidin, avidin and antibodies anti-digoxigenin are commercially available from companies such as Vector Laboratories (Burlingame, Calif.) and Boehringer Mannheim (Indianapolis, Ind.). In another embodiment, the antibodies or streptavidin can be conjugated to quantum dot with superior and more stable fluorescence emission (Wu et al., Nature Biotechnol., 21:41-46, 2003).

[0038] The enzyme in the conjugated antibodies and streptavidin can be detected through a calorimetric reaction by providing a substrate for the enzyme. In the presence of various substrates, different colors are produced by the reaction, and these colors can be visualized to separately detect multiple probes. Any substrate known in the art may be used. Preferred substrates for alkaline phosphatase include 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT). The preferred substrate for horseradish peroxidase is diaminobenzoate (DAB). Those skilled in the art understand that other enzymatic activities also can be used.

[0039] The conditions to carry out *in situ* hybridization to achieve accurate and reproducible results are described. Those of ordinary skill in the art of nucleic acid hybridization will recognize that factors commonly used to control the stringency of hybridization include formamide concentration or other chemical denaturant reagents, salt concentration or variable ionic strength, hybridization temperature, detergent concentration, pH and the presence or absence of chaotropic agents. These stringency factors can be modulated to thereby control the stringency of hybridization of the oligonucleotide probes for the chimeric RNA. Optimal stringency for an assay may be experimentally determined by examination of each stringency factor until the desired degree of discrimination is achieved.

[0040] Other conditions that have to be controlled for optimal *in situ* hybridization are for example the use of chemical agent to block non-specific binding of the probe to components present in the biological samples others than the target chimeric RNAs. The blocking agent may include, but is not limited to, RNA, DNA or oligonucleotides without a label. The blocking agent incorporated in the hybridization solution will suppress the non-specific binding of the labeled probe, and hence, increase the signal to noise ratio of the assay. In yet another aspect of the disclosure, the probe has a sequence complementary to the sequence of the sense or antisense mitochondrial chimeric RNAs (see SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5 and SEQ ID NO 6).

[0041] Fixation of the biological sequence is also an important aspect of *in situ* hybridization that has to be determined experimentally. Highly cross-linking fixative such as glutaraldehide is not recommended since it may block the access of the probe to the target sense mitochondrial chimeric RNA or antisense mitochondrial chimeric RNA. The preferred method of this disclosure is to fix the biological sample with formalin, although frozen samples are also preferred. To expose the sense mitochondrial chimeric RNAs or the antisense mitochondrial chimeric RNA to the labeled probe, additional procedures can be used. For example, the biological sample can be digested with proteinase K to remove proteins that can block the access of the probe to the target chimeric RNAs. Treatment of biological samples with proteinase K or other proteases prior to *in situ* hybridization is well known for those artisans in the art.

[0042] As described before, the presence of long inverted repeats in the chimeric RNA and in the antisense chimeric RNAs, induces the formation of highly stable double stranded structures. These structures together with the secondary structures of the single strand region of the chimeric RNAs may constitute barriers for the access of the probe to the target chimeric RNAs. Therefore, in another aspect of the disclosure, the biological sample is treated with 0.2 M HCl for 10 minutes at room temperature to denature the chimeric RNA. Then the sample is rapidly neutralized by several washes with a buffer solution at pH 7.4 before applying the *in situ* hybridization protocol described herein. Aided by no more than routine experimentation and the disclosure provided herein, those of skilled in the art will easily be able to determine suitable hybridization conditions for performing assays utilizing the methods and compositions described herein. Suitable in *situ* hybridization conditions are those conditions suitable for performing an *in situ* hybridization procedure. Thus, suitable *in situ* hybridization conditions will become apparent using the disclosure and references herein; with or without additional routine experimentation.

[0043] The localization of the sense mitochondrial chimeric RNAs as determined by *in situ* hybridization may have important information for prognosis and management of the patient with cancer. In tumor cells, the sense mitochondrial chimeric RNA is found mostly in the cytoplasm in close association with late endosomes/lysosomes. However, localization in the nucleolus is also found in certain cells. In tumor cells present in human biopsies, the hybridization signal reveals that the sense mitochondrial chimeric RNA is in the cytoplasm only, or in the cytoplasm and the nucleolus or in the cytoplasm and the nucleus. Therefore the different localizations may have an important prognostic value. In a preferred embodiment, panel of human biopsies, for example from breast, colorectal or prostate tumors, may be studied by *in situ* hybridization to detect the chimeric RNA. Together with the positive hybridization signal (independent on how the probe

was labeled), the intracellular localization (only cytoplasm, cytoplasm and nucleus or cytoplasm and nucleolus) should be established in each tumor and the results compared with the survival of each patient.

[0044] A mixture of individual cells containing normal and/or tumor cells can be subjected to hybridization in suspension with oligonucleotide probes labeled with fluorochromes and complementary to the sense mitochondrial chimeric RNA and to the antisense mitochondrial chimeric RNA. For example the probe or probes targeted to the sense mitochondrial chimeric RNA can be labeled with rhodamine, and the probe or probes targeted to the antisense mitochondrial chimeric RNA can be labeled with Alexa 488. After hybridization and washing under the conditions described before, the cells can be analyzed by intracellular labeling flow cytometry.

[0045] It is preferred to use *in situ* hybridization since the information obtained about the specific localization of the chimeric RNA in the tumor cell provides important additional information of prognosis.

[0046] Alternative molecular methods can be used to detect the expression of the chimeric RNA and differential expression of the sense and antisense chimeric RNA in normal, pre-cancer and cancer cells. These alternative methods include, but are not limited to, Northern blot, dot blot, oligonucleotide arrays for the chimeric RNA and the antisense chimeric RNAs, amplification of the RNA by RT-PCR, amplification of the RNA by *in vitro* transcription mediated amplification or TEA, S 1 or ribonuclease protection assays, etc.

[0047] In one embodiment of the present disclosure, the sense mitochondrial chimeric RNA can be detected for diagnostic purposes with a probe obtained by amplification of a region that contains part of the 5' end of the 16S ribosomal RNA and a partial or full region of the inverted repeat. As shown in Fig. 1, the reverse primer can be, for example, primer 1 (SEQ ID NO 139), and the forward primers can be primers 3, 4, 5, 6 or 7 (SEQ ID NOS 129, 116, 106, 102, 63). Primers located at other positions can also be used and they are easily designed by those skilled in the art. In another aspect of this disclosure, the cDNA can be synthesized with an enzyme with reverse transcription activity and random primers such as hexamers or longer, familiar to those skilled in the art.

[0048] The amplicons of 210, 350, 500 or 850 by obtained, or of other sizes resulting by using primers located at other positions, can be detected by gel electrophoresis in agarose gel or polyacrylamide gels (Sambrook *et al.,* 1989) and staining with ethidium bromide or other intercalating dyes. The amplicons can be purified according to the manufacturer's instructions.

[0049] The detection of the mitochondrial chimeric RNA can be carried out by Northern blot analysis (Sambrook *et al.,* 1989). After separation of the RNAs in an agarose gel, the fragments are transferred to a membrane (nitrocellulose or Nylon) by procedures well known to those skilled in the art (Sambrook *et al.,* 1989). To probe the membranes, a fragment of 250 by corresponding to positions 1000 to 125 of the sense mitochondrial chimeric RNA can be amplified. The amplicon is purified (Wizard, Promega) according to the manufacture s instructions and 10 nanograms are used as template for a second amplification. This amplification is carried out with the standard mixture of PCR (Invitrogen) plus 5 micro Curie of ^{32}P dCTP (Amersham). The radioactive amplification fragment is denatured by incubation at 95°C for 10 minutes and the denatured probe was added to the hybridization mixture. The membranes are hybridized for 16 hours at 65°C and then washed twice with 2 times SSC buffer, twice with 0.5 SSC at 60°C and 0.2 SSC at 45°C (Sambrook *et al.,* 1989). The washed membrane was exposed to X-ray film overnight at -70°C (Sambrook *et al.,* 1989). The hybridization signal on the membrane corresponds to a major component of about 2,400 nucleotides which is the size corresponding to the 16S ribosomal RNA (1559 nucleotides) plus the inverted repeat of 815 nucleotides.

[0050] In another embodiment of the disclosure, part of the sense mitochondrial chimeric RNA can be detected after ribonuclease digestion of total RNA extracted from cells or tissues. The double stranded structure or the stem of the sense mitochondrial chimeric RNA is resistant to digestion with ribonuclease A. Total RNA from cells or tissues extracted with TriZol (Invitrogen) is dissolved in a small volume of 2 times SSC. The solution is incubated with ribonuclease A (Sigma) at a final concentration of 50 micrograms per ml. After 30 min at 25°C, the RNA resistant to the nuclease is extracted with TriZol and precipitated with isopropanol at -20°C overnight. The RNA resistant to the nuclease is dissolved in distilled DEPC-treated water and used as template for RT-PCR amplification. This amplification, carried out with primers targeted to positions 55 and 790 of the 16S ribosomal RNA, yields a fragment of about 730 base pairs with a sequence that shows 100% identity with the sequence of the stem of the sense mitochondrial chimeric RNA (SEQ ID NO 1). In contrast, the single strand of the chimeric RNA and corresponding to the 3' half, or the 12S mitochondrial ribosomal RNA, or the 18S ribosomal RNA or the mRNA for GAPDH are totally digested by the treatment with the ribonuclease A, and therefore, no amplification product is obtained when primers targeted to these RNAs are used.

[0051] In another aspect of the disclosure, the stem of the sense mitochondrial chimeric RNA obtained after treatment of total RNA with ribonuclease A can be detected by Northern blot. The RNA resistant to the nuclease and recovered by extraction with TriZol and precipitation with isopropanol, is separated by electrophoresis in an agarose gel. After transfer, the membrane is blotted with the probe described before and used for Northern blot (Sambrook *et al.,* 1989) for the sense mitochondrial chimeric RNA.

[0052] Each set of two or more oligonucleotide probes are preferably labeled with independent detectable moieties so that in an individual cell of the biological sample the sense mitochondrial chimeric RNAs or the antisense mitochondrial chimeric RNAs can be detected. In a preferred embodiment, the oligonucleotide probes which are use to detect the

sense mitochondrial chimeric RNAs or the antisense mitochondrial chimeric RNAs are each labeled with a different hapten. The hapten can be biotin, digoxigenin or fluorescein that can be recognized in the method of *in situ* hybridization with antibodies or streptavidin labeled with different enzymes (e.g. alkaline phosphatase or peroxidase). Alternatively, each oligonucleotide probe of each set of probes can be labeled with independent detectable fluorescent groups. For example, the set of oligonucleotide probes to detect the sense mitochondrial chimeric RNA can be labeled with rhodamine, while the set of oligonucleotide probes to detect the antisense mitochondrial chimeric RNAs can be labeled with Alexa 488. Furthermore, the compositions can be used to determine the localization of the chimeric RNA or the antisense chimeric RNAs in cells of the biological sample. Additionally, compositions of the disclosure can be used to determine the co-localization of the chimeric RNAs or the antisense chimeric RNAs with specific markers of the different cell organelles, by using confocal microscopy analysis.

[0053] The compositions provided herein are deemed particularly useful for the detection and diagnosis of pre-cancer and cancer. The term cancer as used herein includes cells afflicted by any one of the following identified anomalous conditions. These include myeloid leukemia acute or chronic, lymphoblastic leukemia acute or chronic, multiple myeloma, Hodgkin's disease, non-Hodgkin's lymphoma or malignant lymphoma; stomach carcinoma, esophagus carcinoma or adenocarcinoma, pancreas ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, small bowel adenocarcinoma, colorectal carcinomas; hepatocellular carcinoma, hepatocellular adenoma; carcinoids, genitourinary tract such as kidney adenocarcinoma, Wilm's tumor, bladder and urethra carcinoma and prostate adenocarcinoma, testis cancer like seminoma, teratoma, teratocarcinoma, interstitial cell carcinoma; uterus endometrial carcinoma, cervical carcinoma, ovarian carcinoma, vulva and vagina carcinoma, Sertoli-Leydig cell tumors, melanoma, and fallopian tubes carcinoma; lung, alveolar and bronchiolar carcinomas; brain tumors; skin malignant melanoma, basal cell carcinoma, squamous cell carcinoma and Karposi's sarcoma. Also fibrosarcoma, angiosarcoma and rhabdomyosarcoma of the heart and other malignancies familiar to those skilled in the art are included.

Cancer and pre-cancer therapy

[0054] Chemotherapeutic drugs can induce a series of cellular responses that impact on tumor cell proliferation and survival. The best studied of these cellular responses is apoptosis and is evident at the present time that anti-cancer drugs can kill tumor cells by activating common apoptotic pathways. Unfortunately, these drugs also affect rapidly dividing normal cells of the bone marrow, normal hematopoietic and intestinal cells and hair matrix keratinocytes (McKinnell et al., The biological Basis of cancer, 1998; Komarov et al., Science, 285:1733-1737, 1999; Johnstone et al., Cell, 108:153-164, 2002).

[0055] On the other hand, many tumor cells have mutated apoptotic initiator factors, regulatory factors and execution factors of apoptosis, which explain why tumor cells of different cancer types become resistant to a variety of chemotherapeutic drugs and radiation. Mutations have been reported of factors of the intrinsic pathway, post mitochondrial events and the extrinsic pathway of apoptosis (Ramping et al., Science, 275:967-969, 1997; Vogelstein et al., Mature, 408: 307-310, 2000; Teitz et al., Nature Med., 6:529-535, 2000; Reed, J. Clin. Oncol., 17:2941- 2953, 1999; Johnston et al., Cell, 108:153-164, 2002). Therefore, a paradigm of a cancer therapy treatment is a procedure that selectively triggers apoptosis of tumor cells, that does not alter normal proliferating cells and that bypasses the altered or mutated factors of the different apoptotic pathways.

[0056] The compositions of the present invention are based on the discovery that tumor and pre-tumor cells over express the sense mitochondrial chimeric RNA at similar levels of the normal proliferating cells. However, and in contrast with normal proliferating cells, tumor and pre-tumor cells down regulate the expression of the antisense mitochondrial chimeric RNA.

[0057] The structures of these transcripts are shown in Fig. 1 and Fig. 3, and the corresponding sequences in SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5 and SEQ ID NO 6.

[0058] In contrast, and constituting another surprising discovery, pre-tumor and tumor cells over express the sense mitochondrial chimeric RNA and down regulate the expression of the antisense mitochondrial chimeric RNA. The suppression or inhibition of the synthesis of the antisense mitochondrial chimeric RNA in pre-tumor and tumor cells constitutes a novel difference in phenotype between a cancer cell and a normal proliferating cell, which is considered as one of the major embodiments of the present invention. Moreover, tumor cells in human biopsies of different cancer types, also exhibit the same phenotype of cancer cells in culture (Fig. 5A and 5B).

[0059] Although the function of the sense mitochondrial chimeric RNA and the antisense mitochondrial chimeric RNA is not clear, a close correlation exists between the expression of these RNAs and cell proliferation. For example, normal proliferating cells in tissues like liver, kidney and spleen, and defined as such by the expression of the antigens such Ki-67, PCNA or phosphorylated histone H3, over express the sense mitochondrial chimeric RNA as well as the antisense mitochondrial chimeric RNA. In the non-proliferating cells of the same tissues, which do not express Ki-67 or PCNA, the sense mitochondrial chimeric RNA and the antisense mitochondrial chimeric RNA are not expressed. Furthermore, and as illustrated in Fig. 7, human lymphocytes stimulated with the mitogen PHA synthesize DNA and express the proliferating

antigens Ki-67 and PCNA. The stimulated lymphocytes also over express the sense mitochondrial chimeric RNA as well as the antisense mitochondrial chimeric RNA (Fig. 7). In contrast, resting lymphocytes or non-stimulated lymphocytes do not express neither the sense mitochondrial chimeric RNA nor the antisense mitochondrial chimeric RNA.

[0060] The previous finding, which is one fundamental part of the present invention, shows that while normal proliferating cells express the sense and antisense mitochondrial chimeric RNAs, tumor cells express the sense mitochondrial chimeric RNA and down regulate the expression of the antisense mitochondrial chimeric RNA. To understand the function of these RNAs in cell proliferation, cancer cells in culture were treated with antisense oligonucleotides targeted to the sense mitochondrial chimeric RNAs (SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3) or to the antisense mitochondrial chimeric RNA (SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6). The results, constituting another surprising discovery, showed that under these conditions the cells undergo programmed cell death or apoptosis. After treatment with the oligonucleotides complementary to the sense or antisense mitochondrial chimeric RNAs for 6 to 15 hours, between 75 to 96% of the cells undergo apoptosis (Table 2). The changes observed in the treated cells were chromatin condensation, nuclear fragmentation, DNA fragmentation, activation of caspases and altered process of the cell membrane. Control oligonucleotides with 4 or more mismatches or scrambled oligonucleotides did not induce apoptosis. Also, cells were not affected if treated with oligonucleotides targeted to the sense or antisense 12S mitochondrial RNA or targeted to the mRNA or the antisense transcript of the mitochondrial ND1 subunit. In general, oligonucleotides targeted to the antisense mitochondrial chimeric RNA were much more effective, at the same concentration, than oligonucleotides targeted to the sense mitochondrial chimeric RNA. This was an expected result since the tumor cells over express the sense mitochondrial chimeric RNA and therefore it is more difficult to reach a concentration of oligonucleotides inside the cell to interfere with all the copies of this transcript. On the other hand, since tumor cells down regulate the antisense mitochondrial chimeric RNA, it should be easier to interfere with this RNA since there are a lower number of copies per cell.

[0061] The induction of apoptosis is also selective for tumor cells. Resting human lymphocytes or human lymphocytes stimulated for 48 hours with PHA are not affected by the treatment with oligonucleotides complementary to the antisense mitochondrial chimeric RNAs or targeted to the sense mitochondrial chimeric RNA even after overnight treatment and with a high dose of complementary oligonucleotides (15 uM).

[0062] Apoptosis induction by treatment with complementary oligonucleotides targeted to the antisense mitochondrial chimeric RNA (SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6) has been achieved by, but is not limited to, promielocytic leukemia cell HL-60, acute lymphoblastic leukemia MOLT- 4, T-lymphocitic leukemia cells, Jurkat, a T-cell leukemia, Devernelle or B-lymphoma, NSO/2 or myeloma, HeLa cells, DU145, PC- 3, Caco-2, Hep-2 and HepG2. Two cells, MCF/7 (breast carcinoma) and melanoma, that can be considered as a paradigm of treatment-resistant (chemotherapy or radiotherapy) tumor cells undergo apoptosis at a rate of over 80% when treated for 15 hours with complementary oligonucleotides targeted to the antisense mitochondrial chimeric RNA (SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6). A lower apoptotic effect was obtained with oligonucleotides complementary to the sense chimeric RNA (SEQ ID NO 1). As reported before, oligonucleotides with 4 mismatches or scrambled oligonucleotides do not induce cell death.

[0063] Described below are compositions for treating cancer using the sense chimeric RNAs and the antisense chimeric RNAs as a therapeutic target.

[0064] The preferred embodiments, but not limited to, are compositions for treating cancer using oligonucleotides complementary to the antisense chimeric RNAs. The outcome of this treatment is to at least produce in a treated subject a healthful benefit, which in the case of cancer, includes, but is not limited to, remission of the cancer, palliation of the symptoms of the cancer, and control of metastatic spread of the cancer. All such methods involve the induction of apoptosis in the tumor cells and with minor effect in normal cells. Complementary oligonucleotides that target specific RNAs have been used to diminish or abrogate the expression of a large variety of mRNA or the synthesis of the corresponding proteins (e.g. Vickers et al., J. Biol. Chem., 278:7108-7118, 2003). At present, about 42 antisense oligonucleotides with different chemistries are currently being screened as potential drugs (Stephens and Rivers, Burr. Opin. Mol. Therapeut., 5:118-122, 2003) (see also as examples U.S. Pat. Nos. 5,801,154; 6,576,759; 6,720,413; 6,573,050 and 6,673,917).

[0065] In one aspect of this invention, one or more oligonucleotides targeted to the antisense mitochondrial chimeric RNA can be used. The use of two or more complementary oligonucleotides is more effective and shows some degree of synergism.

[0066] The oligonucleotide of the invention may be complementary to the antisense mitochondrial chimeric RNA or to the sense mitochondrial chimeric RNA. The complementary oligonucleotides will bind to the antisense mitochondrial chimeric RNAs or to the sense mitochondrial chimeric RNAs and interfere with their functions. Absolute complementarily, although preferred, is not required. An oligonucleotide sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarily to be able to hybridize with the RNA, forming a stable duplex. The ability to hybridize will depend on both the degree of complementarily and the length of the oligonucleotide. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex. Those skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

[0067] In general, complementary oligonucleotides to hybridize with mRNAs for different proteins are targeted to the 5' untranslated region of the mRNA including the complement of the AUG start codon, or the 3' untranslated region to be more effective. Oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation (see previous references). The sense mitochondrial chimeric RNA and the antisense mitochondrial chimeric RNA are non-coding RNA and therefore the target region of the oligonucleotides can be complementary to any region of these transcripts. The most effective regions are located around the single-stranded segments of the antisense mitochondrial chimeric RNA determined by scanning the sequences of the antisense or the sense mitochondrial chimeric RNA with complementary oligonucleotides designed every 30 nucleotides. Those skilled in the art will understand that other sequences within the complete sequences of the transcripts of SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, and SEQ ID NO 6, are targets to design alternative complementary oligonucleotides.

[0068] The complementary oligonucleotides targeted to the antisense mitochondrial chimeric RNA or to the sense mitochondrial chimeric RNA resulting in the induction of tumor cell death according to the present invention will generally contain backbones different to the natural phosphodiester bonds. The oligonucleotides can have alternate inter nucleoside linkages, comprising, but not limited to, phosphorothioate (Mag et al., Nucleic Acids Res., 19:1437-1441, 1991; and U.S. Pat. No. 5, 644,048), peptide nucleic acid or PNA (Egholm, Mature, 365:566-568, 1993; and U.S. Pat. No. 6,656,687), phosphoramide (Beaucage, Methods Mol. Biol., 20:33-61, 1993), phosphorodithioate (Capaldi et al., Nucleic Acids Res., 28:E40, 2000). Other oligonucleotide analogs include, but are not limited to, morpholino (Summerton, Biochim. Biophys. Acta, 1489:141-158, 1999), locked oligonucleotides (Wahlestedt wt al., Proc. Natl. Acad. Sci., US, 97:5633-5638, 2000), peptidic nucleic acids or PNA (Nielsen et al., 1993; Hyrup and Nielsen, 1996) or 2-o-(2-methoxy) ethyl modified 5' and 3' end oligonucleotides (McKay et al., J. Biol. Chem., 274:1715-1722, 1999). The nucleic acids may contain any combination of deoxyribo- and ribonucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. The complementary oligonucleotides according to the invention may comprise at least one modified base moiety which is selected from the group including, but not limited, to 5- fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1- methylquanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2- methylquanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7- methylquanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2- thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5- methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl- 2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid. methylester, uracil-5-oxyacetic acid (v), 5-methyl-2- thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

[0069] The complementary oligonucleotides may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

[0070] In another embodiment of the present invention, the complementary oligonucleotides are designed to hybridize with any region of the antisense mitochondrial chimeric RNA or to any region of the sense mitochondrial chimeric RNA. The complementary oligonucleotides should be at least ten nucleotides in length, and are preferably complementary oligonucleotides ranging from 10 to about 50 nucleotides in length. In specific aspects, the complementary oligonucleotide is at least 12 nucleotides, at least 18 nucleotides, at least 22 nucleotides, at least 30 nucleotides, at least 50 nucleotides.

[0071] It is important to consider, for *in vitro* as well as for *in vivo* experiments, utilizing controls that distinguish between antisense interference with the function of the antisense mitochondrial chimeric RNA or the sense mitochondrial chimeric RNA with nonspecific biological effects of antisense or complementary oligonucleotides. Therefore, the design of the oligonucleotides has to avoid the presence in the sequence of CpG tracks, 5' GGGG tracks and other sequences that have toxic effect in animal cells as reported in US Pat. No. 6,673,917. Also the presence of the sequence 5' CGTTA was avoided for the non-antisense effect that was reported (Tidd et al., Nucleic Acids Res., 28:2242-2250, 2000).

[0072] In another embodiment of the present invention, the complementary oligonucleotides targeted to the antisense mitochondrial chimeric RNAs or targeted to the sense mitochondrial chimeric RNAs are used as therapeutic agents to animals or to patients having cancer can induce sensitivity to anti-cancer therapeutic drugs and radiation. Induced sensitivity, also known as sensitization or hypersensitivity, can be measured in tumor cells showing tolerance to anti-cancer therapeutic or radiation. The anti-cancer drugs comprise those already known in the art and in use or as-yet undiscovered drugs. Among the conventional chemotherapeutic drugs are alkylating agents, anti-metabolite, antibiotics and anti-microtubule agents. Some examples of these drugs are cisplatin, methotrexate, doxorubicin dactinomycin, mitomycin, cyclophosphamide, etc.

[0073] Together with or after the treatment of an animal or a patient having cancer with complementary oligonucleotides targeted to the antisense mitochondrial chimeric RNA and/or the sense mitochondrial chimeric RNA, the patient can be treated with radiotherapy, wherein said radiotherapy includes ultraviolet radiation, gamma radiation, alpha particles, beta particles, X-ray and electron beams.

[0074] In another aspect of this disclosure, interference with the function of the antisense mitochondrial chimeric RNA

or the sense mitochondrial chimeric RNA to induce tumor cell death can be achieved by RNA interference or RNA silencing. Over the last six years RNA interference (RNAi) has emerged as a novel and promising approach for gene silencing in mammalian cells (Elbashir et al., Mature, 411:494498, 2001; McManus et al., Nature Rev. Genet., 3:737-747, 2002). Synthetically synthesized double stranded RNA molecules of about 19 to 21 nucleotides in length hybridize specifically to their complementary target mRNA, leading to degradation of the mRNA and subsequent protein knockdown. Several different genes have been silenced successfully by small interfering RNA or siRNA (Lu et al., Curr. Opin. Mol. Ther., 5:225-234, 2003.; Wacheck et al., Oligonucleotides, 13:393-400, 2003). Therefore, synthetic double stranded RNA of about 19 to 21 nucleotides targeted to the antisense mitochondrial chimeric RNA or to the sense mitochondrial chimeric RNA can be used to degrade these transcripts and induce tumor cell death. Those familiar with the art will understand that the sequence of the siRNA has to be complementary to any region of the antisense mitochondrial chimeric RNAs or to the sense mitochondrial chimeric RNAs (SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, and SEQ ID NO 6).

[0075] In another embodiment of the disclosure, ribozymes can be used to interfere with the antisense mitochondrial chimeric RNA or with the sense mitochondrial chimeric RNA to induce tumor cell death. The sequence of the ribozyme has to be designed according to the sequence of the antisense mitochondrial chimeric RNA (SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6) or the sense mitochondrial chimeric RNA (SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3) to cleave specific regions of the transcript that are more efficient to trigger tumor cell death. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA (Ross), Curr. Biology, 4:469-471, 1994). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well-known catalytic sequence responsible for mRNA cleavage, and described in U.S. Pat. No. 5,093,246. As such, within the scope of the invention hammerhead ribozyme molecules are engineered that specifically and efficiently catalyze endonucleolytic cleavage of the antisense mitochondrial chimeric RNAs or the sense mitochondrial chimeric RNAs. The construction and production of hammerhead ribozymes is well known in the art and it was described (Haseloff et al., Gene, 82:43-52, 1989). Ribozymes of the present invention also include RNA endoribonucleases (Zang et al., Science, 224:574-578, 1984).

[0076] Gene therapy refers to treatment or prevention of cancer performed by the administration of a nucleic acid to a patient who has cancer or in whom prevention or inhibition of cancer is desirable. In this embodiment of the present disclosure, the therapeutic nucleic acid produced intracellularly is a complementary RNA targeted to the antisense mitochondrial chimeric RNA or to the sense mitochondrial chimeric RNA that mediates the therapeutic effect by interfering or inhibiting the function of these mitochondrial transcripts, inducing tumor cell death. Therefore, one preferred approach is to utilize a recombinant DNA construct in which the transcription of the antisense RNA is placed under the control of strong promoters of RNA polymerase II or III. Expression of the sequence encoding the complementary RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters include but are not limited to SV40 early promoter region (Benoist and Chambon, Mature, 290:304-310, 1981), the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A., 78:1441- 1445, 1981), the regulatory sequences of the metallothionein gene (Brinster et al., Mature, 296:39-42, 1982), the promoter of Rous sarcoma virus (Yamamoto et al., Cell, 22:787-797, 1980), etc. The recombinant DNA construct to produce the complimentary RNA can be a viral vector which includes, but is not limited to adenovirus vector, adeno-associated virus vector, herpes simplex virus vector, vaccinia virus vector and retrovirus vectors. The vector is introduced in the target tumor cells, in a pharmaceutical composition, using methods familiar to those skilled in the art.

[0077] Pharmaceutical compositions of the disclosure comprising an effective amount of a complementary nucleic acid (complementary oligonucleotides, siRNA, ribozymes or viral vectors) in a pharmaceutically acceptable carrier, that can be administered to a patient having cancer to interfere with the function of the antisense mitochondrial chimeric RNA or the sense mitochondrial chimeric RNA and induce apoptosis of the tumor cells. The complementary nucleic acids may be formulated in a pharmaceutical composition, which may include carriers, diluents, buffers, preservatives, surface active agents, polyethylenimide (PEI), liposomes or other lipid formulation known in the art. The pharmaceutical composition may be administered by topical application, oral, parenteral or rectal administration. Parenteral administration includes intravenous, subcutaneous, intraperitoneal or intramuscular injection or pulmonary administration by inhalation or insufflation.

[0078] The compositions of the present disclosure can be utilized for therapeutics, diagnostics, prophylaxis and as research reagents and kits.

[0079] The compositions provided herein are deemed particularly useful for the treatment of cancer. The term cancer as used herein includes cells afflicted by any one of the following identified anomalous conditions. These include myeloid leukemia acute or chronic, lymphoblastic leukemia acute or chronic, multiple myeloma, Hodgkin's disease, non-Hodgkin's lymphoma or malignant lymphoma; stomach carcinoma, esophagus carcinoma or adenocarcinoma, pancreas ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, small bowel adenocarcinoma, colorectal carcinomas; hepatocellular carcinoma, hepatocellular adenoma; carcinoids, genitourinary tract such as kidney adenocarcinoma, Wilm's

tumor, bladder and urethra carcinoma and prostate adenocarcinoma, testis cancer like seminoma, teratoma, teratocarcinoma, interstitial cell carcinoma; uterus endometrial carcinoma, cervical carcinoma, ovarian carcinoma, vulva and vagina carcinoma, Sertoli-L-eydig cell tumors, melanoma, and fallopian tubes carcinoma; lung, alveolar and bronchiolar carcinomas; brain tumors; skin malignant melanoma, basal cell carcinoma, squamous cell carcinoma and Karposi's sarcoma. Also fibrosarcoma, angio sarcoma and rhabdomyosarcoma of the heart and other malignancies that are familiar to those skilled in the art. Thus, the term "cancerous cell" as provided herein, includes a cell afflicted by any one of the above-identified conditions.

[0080] The following examples serve to describe the manner of using the above-described invention as well as to set forth the best manner for carrying out various aspects of the invention. It is understood that in no way these examples meant to limit the scope of this invention, but rather they are presented for illustrative purposes.

EXAMPLE 1

Isolation and sequence of the sense mitochondrial human chimeric RNA. (Fig. 1A, SEQ ID NO 1)

[0081] Initial experiments indicated that the putative human sense mitochondrial chimeric RNA contained a more complex and stable secondary structure than the mouse chimeric RNA (Villegas et al., DNA & Cell Biol., 19:579-588, 2000; Villegas et al., Nucleic Acids Res., 30:1895-1901, 2002). Therefore, and based in the mouse mitochondrial chimeric RNA secondary structure, a theoretical human sense mitochondrial chimeric RNA secondary structure was deduced (Fig. 1A). The theoretical human transcript contained the complete sequence of the sense 16S mitochondrial RNA joined by the 5' end to a fragment of the antisense 16S mitochondrial RNA forming a loop of unknown length (Fig. 1A). The segment of the antisense 16S mitochondrial RNA was fully complementary to the sense 16S mitochondrial RNA and therefore corresponded to an inverted repeat joined to the 5' end of the sense 16S transcript. Based on this structure, primers were designed to amplify this putative transcript by RT-PCR. One reverse primer was at position 11 to 31 from the 5' end of the human sense 16S mitochondrial RNA or at the beginning of the theoretical loop (primer 1, Fig. 1A) (SEQ ID NO 139). The sequence of the forward primer used was that of positions 213-234 of the sense 16S mitochondrial RNA, and corresponds to primer 3 in Fig. 1A. Amplification of RNA from several human tissues and cells including HeLa, HL-60, Du145, MCF/7 and human lymphocytes stimulated with PHA (see Example 7) by RT-PCR using primers 1 and 3 (Fig. 1A), yielded a unique amplicon of about 210 bp (Figure 2). RT-PCR was carried out as described before (Villegas et al., DNA & Cell Biol., 19.579-588, 2000; Villegas et al., Nucleic Acids Res., 30:1895-1901, 2002). The amplicons from each human tissue or cells were cloned and both strands were sequenced. In all cases, an identical sequence of 216 bp was obtained, containing an inverted repeat of 184 nucleotides joined to the first 31 nucleotides of the 5' end of the sense 16S mitochondrial RNA. Then, we determined if the inverted repeat was longer than 184 nucleotides and extended further toward the 5' end of the antisense 16S mitochondrial RNA (Fig.1A). The cDNA from HeLa or other cells described before was amplified between the reverse primer 1 positioned at the loop as described before, and primers 4 to 7 to walk toward the 5' end of the putative longer inverted repeat (Fig. 1A). By using this approach, amplification fragments of approximately 500, 700 and 800 bp were obtained when primer 1 was used in combination with primers 4, 5 and 6, respectively. On the other hand, when the cDNA was amplified between primer 1 and primer 7 no amplification product was obtained, suggesting that the 5' end of the inverted repeat was between primers 7 and 8 (see below). The complete sequence of the amplicon of 800 bp reveals an inverted repeat of 769 nucleotides joined to the first 31 nucleotides of the sense 16S mitochondrial RNA (SEQ ID NO 1) (Fig.1A). The sequence at the 3' end of the inverted repeat joined to the sense 16S mitochondrial RNA was identical to that found in the same region of the amplicon of 216 bp. This is important because it indicates that in both cases we were amplifying the same RNA. In addition, the sequence showed that 50 nucleotides of the 3' end of the antisense 16S mitochondrial RNA was missing in the inverted repeat of the sense mitochondrial chimeric RNA. Altogether, these results suggest that the double stranded structure formed between the inverted repeat and the sense 16S mitochondrial RNA begins at position 51 of the latter, and forms a putative loop of nucleotides.

[0082] To confirm the size of the loop, human cDNA was amplified by PCR between the forward primer 2 positioned at the 3' end of the inverted repeat and primer 3, which is also reverse at positions 213-234 of the sense 16S mitochondrial RNA (Fig.1A). An amplicon of approximately 240 bp was obtained and the sequence showed that the first 234 nucleotides of the sense 16S mitochondrial RNA were joined to the last 25 nucleotides of the 3' end of the inverted repeat. The sequence of the 25 nucleotides of the inverted repeat was fully complementary to the sense 16S mitochondrial RNA from positions 51 to 75 (Fig. 1A).

[0083] If the sequence of the amplicon obtained with primers 1 and 6 and the sequence of the amplicon obtained with primers 2 and 3 are assembled as contigs, the emerging structure of the human sense mitochondrial chimeric RNA confirmed a loop of 50 nucleotides and a double stranded structure of at least 769 bp (Fig. 1A) (see also SEQ ID NO 1).

[0084] Since double stranded RNA is not digested by RNase A, the stem of the human sense mitochondrial chimeric RNA should be resistant to this enzyme. On the other hand, the loop or the 3' region of the sense 16S mitochondrial

RNA strand that extended beyond the double stranded structure should be digested by the enzyme. RNA from HeLa or other cells was digested with RNase A (50 ug per ml), followed by phenol extraction, and the nuclease-resistant material was recovered by ethanol precipitation. The cDNA from the digested RNA was then amplified by PCR using the primers showed in Fig. 1A. The amplicon of about 800 bp obtained with primers 1 and 6 was not amplified after RNase A digestion indicating that the loop was digested by the enzyme. The same was true with the amplicon of 360 bp obtained with primers 10 and 11 as indicated in Fig. 1A. Altogether, these results indicated that the loop as well as the 3' region of the sense mitochondrial chimeric RNA that extends beyond the stem, were digested by the enzyme. On the other hand, amplification of the 750 bp amplicon, corresponding to the double stranded structure of the sense mitochondrial chimeric RNA and obtained with primers 8 and 6, was not affected by the RNase A digestion. The sequence of the double strand fragment resistant to ribonuclease digestion was identical with the expected sequence of the stem. The same results were obtained after digestion of total RNA from HL- 60 cells or other human cells.

[0085] To determine the 5' end of the inverted repeat of the sense mitochondrial chimeric RNA, the stem of the transcript obtained after RNase A digestion was used for 5' RACE analysis. The 5' end determination of the inverted repeat was carried out according to the manufacturer's instructions (Invitrogen). The results indicated that the inverted repeat extends for 46 additional nucleotides from the 5' end of the amplicon obtained after amplification of the sense mitochondrial chimeric RNA with primers 1 and 6. In summary, the inverted repeat of 815 nucleotides is joined to the 5' end of the first 865 nucleotides of the 16S mitochondrial RNA. The sequence of this transcript showed 99.8% identity with the human 16S mitochondrial gene (H and L strand) (SEQ ID NO 1). The 5' ends of both extremes of the double stranded stem were confirmed by 5' RACE.

[0086] The above results indicated that the sense mitochondrial chimeric RNA contained a stem or double stranded structure of 815 base pair and a loop of 50 nucleotides. However, these results do not prove that the inverted repeat is joined to the complete 16S sense mitochondrial RNA. The use of conventional approaches such as synthesis of the complete cDNA from the 3' end is useless, since the double stranded structure of the transcript represents an insurmountable problem to reverse transcriptases, including Tth (Myers and Gelfand, Biochemistry, 30:7661- 7666, 1991). If the inverted repeat of 815 nucleotides is joined to the 1559 nucleotides of the 16S mitochondrial RNA one would expect a transcript of 2.3 Kb. Northern blot analysis of total RNA from HeLa, HL-60 and MCF/7 cells were carried out with a probe labeled with ^{32}P and targeted to the double stranded structure of the sense mitochondrial chimeric RNA. The results revealed a band of about 2.4 Kb, besides a band of 1.6 Kb, corresponding to the sense mitochondrial chimeric RNA and the sense 16S mitochondrial RNA, respectively. If the RNA was digested with RNase A prior to the Northern blot, a single hybridization band of approximately 0.8 Kb was obtained, which corresponds to the size of the stem of the sense mitochondrial chimeric RNA. Altogether, these results strongly demonstrated that the sense mitochondrial chimeric RNA contained an inverted repeat of 815 nucleotides joined to the 5' end of the complete sense 16S mitochondrial RNA, and corresponding to SEQ ID NO 1.

[0087] It is possible to specifically detect the junction region between the inverted repeat and the sense 16S mitochondrial RNA, using an oligonucleotide probe. The probe has to include 7 to 10 nucleotides at each side of the joining point between the 3' end of the inverted repeat and the beginning of the sense 16S mitochondrial RNA. This oligonucleotide can be used for *in situ* hybridization or amplification by RT- PCR or any other methods familiar to those skilled in the art to detect this novel RNA.

[0088] The sense mitochondrial chimeric RNA is present in normal proliferating cells (human foreskin keratinocytes, spleen, lymphocytes stimulated with PHA, mouse embryos), in pre-cancer cells (keratinocytes transformed with HPV 16 or 18, MT-2 cells transformed with HTLV-1) and in tumor cells. It is not present in normal resting cells. A summary of these results is presented in Table 1 (in Example 4).

EXAMPLE 2.

Human keratinocytes transformed with papilloma virus synthesize a novel sense mitochondrial chimeric RNA (Fig. 1B, SEQ ID NO 2).

[0089] Human foreskin keratinocytes (HFK) were transformed by incubation with a lysate of cells previously infected with the human papilloma virus 16 (HPV 16). The cells were cultured with 3 parts of K-SFM, one part of DMEM medium (Invitrogen), 5 ng/ml of EGF, 50 ug/ml of pituitary extract and 10% calf fetal serum. The culture conditions were 37°C and 5% CO_2. After 24 hours of infection, the transformed HFK were transferred to a new flask and grown under the same conditions. After this time the cells (HFK698) were successively transferred to new culture flasks every 3 days using a split ratio of 1:3 to 1:4. After passage 19th the cells (HFK698 transformed with HPV 16) were harvested as described (Heusen, Biochim. Biophys. Acta, 1288:F55-F78, 1996), collected by centrifugation at 300x g for 10 min and washed twice with saline phosphate buffer (PBS). Total RNA was extracted from the washed cells with Trizol (Invitrogen). About 200 nanograms of RNA were used to synthesize the cDNA with random hexamers as described in Example 1. The cDNA was amplified by PCR using the reverse primer 1 and the forward primer 3 as described in Fig. 1A. This

amplification protocol yielded the expected amplicon of 210 bp where the first 31 nucleotides of the sense 16S mitochondrial RNA are joined to the inverted repeat of 184 nucleotides as described before in Example 1. Electrophoresis analysis of the amplification products revealed the presence of the amplicon of 210 base pairs corresponding to the sense mitochondrial chimeric RNA, plus another amplification fragment of about 150 base pairs as shown in Fig. 2. The complete sequence of this new fragment (SEQ ID NO 2) showed that the initial 31 nucleotides from the 5' end of the sense 16S mitochondrial RNA are joined to an inverted repeat of 121 nucleotides, which is shorter by 63 nucleotides if compared with the inverted repeat of the sense mitochondrial chimeric RNA of SEQ ID NO 1. This shorter inverted repeat generates a longer loop of 96 nucleotides (Fig. 1B) in the structure of the mitochondrial chimeric RNA. The remainder of the sequence is identical to SEQ ID NO 1. This novel sense mitochondrial chimeric RNA is not present in SiHa cells (Fig. 4A), which are tumorigenic cells transformed with HPV 16, nor in normal proliferating human cells like human lymphocytes stimulated with PHA (see Example). Similar results were obtained with HFK transformed with HPV 18 or 18Nco cells. The cells transformed or immortalized (but not tumorigenic) with HPV 16 or HPV 18 are considered as pre-malignant cells and therefore the novel sense mitochondrial chimeric RNA is a new potential marker for pre-malignant cells.

[0090] Since the sequence of the 3' end of the inverted repeat of SEQ ID NO 2 joined to the 16S mitochondrial RNA is different from the same region of SEQ ID NO 1, an oligonucleotide probe can be used for the specific detection of this transcript. The probe has to include 7 to 10 nucleotides at each side of the joining point between the 3' end of the inverted repeat and the beginning of the sense 16S mitochondrial RNA, such as the oligonucleotide of SEQ ID NO 7. This oligonucleotide can be used for *in situ* hybridization or amplification by RT-PCR or any other methods familiar to those skilled in the art to detect this novel and specific marker of pre-cancer cells.

EXAMPLE 3:

Cells transformed with HTLV-1 induce the expression of a third novel sense mitochondrial chimeric RNAs (Fig. 1C, SEQ ID NO 3).

[0091] Human MT-2 cells transformed with HTLV-1 were cultured as described (Kobayashi et al., EMBO J., 3:1339-1343, 1984). The cells were harvested, centrifuged at 300 x g for 10 min and washed twice with PBS. The final cell pellet was extracted with Trizol as described in Example 1. The cDNA was synthesized with random hexamers using the RNA as template and the cDNA was amplified by PCR using the reverse primer 1 and the forward primer 3 as described in Fig. 1A. As described before, this amplification protocol yields an amplicon of 210 base pairs that contains the first 31 nucleotides of the sense 16S mitochondrial RNA joined to an inverted repeat of 184 nucleotides which corresponds to the sense mitochondrial chimeric RNA as described in Example 1. Electrophoresis analysis of the amplification products revealed, besides the presence of the already discussed amplicon of 210 base pairs, a band of about 150 base pairs (see Fig. 2). The sequence of the amplicon of 150 base pairs is identical to the sequence of the amplicon described in Example 2, corresponding to a second sense mitochondrial chimeric RNA expressed in cells transformed with HPV 16 or HPV 18 (SEQ ID NO 2). In addition, a new amplification product was found of about 100 bp (Fig. 2). The sequence of this third amplicon revealed an inverted repeat of 61 nucleotides joined to the 5' end of the sense 16S mitochondrial RNA and generating a loop of 167 nucleotides (Fig. 1C; SEQ ID NO 3). This novel amplicon was not present in normal cells, in tumor cells and in cells transformed with HPV 16 or 18. Therefore, this new sense mitochondrial chimeric RNA is a potential marker of cells transformed with the oncogenic retrovirus HTLV-1.

[0092] Since the sequence of the 3' end of the inverted repeat of SEQ ID NO 3 joined to the 16S mitochondrial RNA is different from the same region of SEQ ID NO 1 and SEQ ID NO 2, an oligonucleotide probe can be used for the specific detection of this transcript. The probe has to span between 7 to 10 nucleotides at each side of the joining point between the 3' end of the inverted repeat and the beginning of the sense 16S mitochondrial RNA, such as an oligonucleotide of SEQ ID NO 8. This oligonucleotide can be used for *in situ* hybridization or amplification by RT-PCR or any other methods familiar to those skilled in the art to detect this specific marker of cells transformed with a retroviral oncogenic virus.

EXAMPLE 4.

Structure of the human antisense mitochondrial chimeric RNA.

[0093] Our initial experiments indicated that a second family of chimeric RNAs corresponding to the antisense mitochondrial chimeric RNA was present in some of the cell studies. To establish the structure of the human antisense mitochondrial chimeric RNA, the strategy used for the sense mitochondrial chimeric RNA was employed (Fig. 1). The theoretical antisense mitochondrial chimeric RNA contained a fragment of the sense 16S mitochondrial RNA as an inverted repeat joined to the 5' end of the antisense 16S mitochondrial RNA. The latter RNA is transcribed from the L-

strand of the mitochondrial DNA and corresponds to the 16S mitochondrial gene (Fig. 3). To amplify this RNA, a reverse primer was hybridized close to the 5' end of the antisense 16S mitochondrial RNA and forward primers were hybridized at different positions of the putative fragment of the inverted repeat (Fig. 3). Total RNA from human lymphocytes stimulated with PHA for 48 hours was used as template. The cDNA was synthesized with random hexamers as described in Example 1. Amplification of the cDNA by PCR was carried out with the reverse primer positioned close to the beginning of the 5' end of the antisense 16S mitochondrial RNA (primer 1, Fig. 3) and different forward primers positioned on the inverted repeat (Fig. 3). Only three major amplicons were obtained which differed in the size of the inverted repeat and the size of the loop. These amplicons were purified and sequenced. One of these antisense mitochondrial chimeric RNAs contains an inverted repeat of 365 nucleotides and a loop of 17 nucleotides (SEQ ID NO 4). Another RNA contains a loop of 96 nucleotides and an inverted repeat of 189 nucleotides (SEQ ID NO 5). Yet, another species of the antisense mitochondrial chimeric RNA contains an inverted repeat of 296 nucleotides and a loop of 451 nucleotides (SEQ ID NO 6). The sequences of all three antisense mitochondrial chimeric RNAs were 99.8 percent homologous with the sequence of the mitochondrial DNA gene (H and L strand).

[0094] The results, which will be presented in the following examples indicate that there is a major difference between pre-tumor and tumor cells and normal proliferating cells with respect to the expression of the antisense mitochondrial chimeric RNA. All proliferating cells over express the sense mitochondrial chimeric RNA. However, while normal proliferating cells also express the antisense mitochondrial chimeric RNAs, these transcripts are down regulated in tumor cells. Non-proliferating or resting cells do not express either mitochondrial chimeric RNAs. Therefore, the differential expression of these RNA represents a novel and powerful marker of carcinogenesis, which can be detected by *in situ* hybridization, Northern blot analysis, RT-PCR or TMA or other techniques known by one skilled in the art.

[0095] A summary of the differential expression of the sense and antisense mitochondrial chimeric RNAs is shown in Table 1.

Table 1.
Expression of the chimeric RNAs in different type of cells.

Chimeric RNAs	Normal Resting	Normal Proliferating	Transformed with HPV	Transformed withe HTLV-1	Cancer
SEQ ID NO 1	--	+++++	+++++	+++++	+++++
SEQ ID NO 2	--	--	++++	++++	--
SEQ ID NO 3	--	--	--	++++	--
SEQ ID NO 4	--	+++++	+/-	+/-	+/-
SEQ ID NO 5	--	+++++ +/-	+/-	+/-	+/-
SEQ ID NO 6	--	+++++	+/-	+/-	+/-

+ and -: relative level of expression by In situ hybrization

EXAMPLE 5.

Tumor cells lines over express the sense mitochondrial chimeric RNA (SEQ ID NO 1) and down regulate the expression of the antisense mitochondrial chimeric RNA (SEQ ID NOS 4,5 and 6).

[0096] *In situ* hybridization was used to determine the expression of the sense mitochondrial chimeric RNA in tumor cell lines in culture. For *in situ* hybridization, adherent tumor cells were cultured in 8-wells chamber slides (Lab-Tek, NUNC) for 24 to 48 hours at 37°C, using the appropriate medium and conditions recommended by American Tissue Culture Collection or ATCC. For non-adherent cells (e.g. HL- 60, Jurkat and Ramos), they were cultured in small flask for 48 hours at 37°C.The cells were recovered by centrifugation at 300 x g for 10 minutes, re-suspended in small volume of PBS and aliquots of 10 to 20 ul were applied on glass slides previously coated with polylysine or an adhesive protein purified from mussels (Burzio et al, Curr. Opin. Biotechnol., 8:309-312,1997). The cells were dried at room temperature for 30 minutes. The cells were washed three times with PBS and fixed with 4% pare-formaldehyde for 10 minutes at room temperature. The slides were then washed three times with PBS for 5 minutes and incubated with 0.2 N HCl for 10 minutes at room temperature. The cells were washed again three times, first with PBS and then with 2X SSC for 10 minutes (2X SSC: 0.3 M NaCl, 30 mM sodium citrate, pH 7.0) (Sambrook et al., 1989) at room temperature. The prehybridization was carried out for 30 minutes at 37°C in a solution containing 4X SSC, 10% dextran sulfate, 150 g/ml yeast tRNA and herring sperm DNA, 50% formamide and 1X Denhardt solution (0.2 mg/ml Ficoll type 400, 0,2 mg/ml polivinylpyrrolidone, 0.2 mg/ml BSA). Hybridization was carried out for 15 hours at 37°C in the same prehybridization mixture containing 3.5 pmoles of probes targeted to the sense and antisense mitochondrial chimeric RNAs. The probes contained 20 or more deoxynucleotides targeted to different regions of the sequence of the sense or antisense mito-

chondrial chimeric RNA (see SEQ ID NOS 98 to 196 and SEQ ID NOS 9 to 97, respectively). The probes were previously labeled at the 3' end with digoxigenin-11-dUTP (Roche) and terminal transferase (Promega) as described before (Villegas et al., DNA & Cell Biol., 19:579-588, 2000). To eliminate the excess of probe, the slides were washed first with 2X SSC for 10 minutes and with 1X SSC for 10 minutes at room temperature. Then the samples were washed with 0.2X SSC for 30 minutes at 45°C and finally, with 0.2 X SSC for 10 minutes at room temperature.

[0097] After hybridization, the cells were incubated for 30 minutes in blocking buffer (1 % BSA, 0,3 % Triton X-100 in PBS) and then incubated for 2 hours at room temperature with anti-digoxigenin monoclonal antibody conjugated to alkaline phosphatase (Roche), previously diluted 1:500 in the blocking buffer. Finally, the slides were washed twice with PBS and the color reaction was carried out with a BCIP/NBT substrate mixture (DAKO) as described before (Villegas et al., DNA & Cell Biol., 19:579- 588, 2000). The same procedure was employed for FISH, using anti-digoxigenin antibodies conjugated with fluorescein or rhodamine.

[0098] As shown in Fig. 4A and 4B, *in situ* hybridization with a probe labeled with digoxigenin corresponding to SEQ ID NO 63 reveals that human tumor cells overexpress the sense mitochondrial chimeric RNA. *In situ* hybridization with the sense probe labeled with digoxigenin and corresponding to SEQ ID NO 64 was negative (Fig. 4A and B) indicating down regulation of the expression of the antisense mitochondrial chimeric RNA. The same results were obtained with oligonucleotide probes targeted to other regions of the sense or antisense mitochondrial chimeric RNA.

EXAMPLE 6.

Tumor cells in human biopsies over express the sense mitochondrial chimeric RNA (SEQ ID NO 1) and down regulate the antisense mitochondrial chimeric RNA (SEQ ID NOS 4, 5 and 6).

[0099] Human biopsies were obtained from pathologists or tissue arrays from DAKO. Most of the samples analyzed were paraffin-embedded and fixed with formalin. Other tissue samples were fixed with Boiun's fixative and yet other samples were fresh frozen tissue sections. The tissue sections of about 4 to 8 μm were fixed on slides previously coated with polylysine or the adhesive polyphenolic protein purified from the mussel *Aulacomya ater* (Burzio et al., Curr. Opin. Biotechnol., 8:309-312, 1997). The paraffin-embedded tissue sections were incubated for 1 hour at 60°C, and the paraffin was removed by three washes with xylol for 15 minutes each time. The sections were air dried and washed four times with PBS. Then the sections were incubated with 0.2 N HCl for 10 minutes at room temperature and then thoroughly washed with PBS. Afterwards, the samples were subjected to *in situ* hybridization with the antisense probes labeled with digoxigenin according to protocol described in Example 4. A parallel section was hybridized with a sense probe corresponding to the same region of the sense mitochondrial chimeric RNA.

[0100] As shown in Fig. 5A, the cells present in tumors of breast, uterine cervix, bladder and lung carcinoma revealed a strong staining with the antisense probes targeted to the sense mitochondrial chimeric RNA, indicating strong presence of the transcript. On the other hand the *in situ* hybridization with the probe targeted to the antisense mitochondrial chimeric RNA was negative, indicating down regulation of this transcript (Fig. 5A). Other tumors also over express the sense mitochondrial chimeric RNA, and down regulate the expression of the antisense mitochondrial chimeric RNA (Fig. 5B).

EXAMPLE 7.

Normal proliferating cells over express the sense and the antisense mitochondrial chimeric RNAs.

[0101] Using the same protocol for *in situ* hybridization described in Examples 5 and 6, the expression of the sense mitochondrial chimeric was determined in proliferating cells. As shown in Fig. 6, HFK cells, spermatogonia, spleen cells and proliferating cells of mouse embryo, showed a strong hybridization signal indicating over expression of the sense mitochondrial chimeric RNA. In contrast, non-proliferating cells such as cells of the brain, muscle and liver show no signal indicating that the sense mitochondrial chimeric RNA is not expressed or is down regulated in these cells.

[0102] However, the surprising result was that when the *in situ* hybridization was carried out with probes targeted to the antisense mitochondrial chimeric RNA, a strong signal was also observed (Fig. 6). Several controls assayed in parallel indicated that the hybridization signal with these probes was not due to an artifact. The hybridization signal disappeared if the *in situ* hybridization was carried out with the labeled probe together with an excess (50 to 100 times) of the same probe but non-labeled with digoxigenin. If previous to the hybridization the samples were incubated with ribonuclease A overnight, the hybridization signal disappeared. Also, no hybridization signal was observed if the hybridization was carried out with a labeled probe targeted to the antisense mitochondrial chimeric RNA with 4 mismatches.

EXAMPLE 8.

Normal human lymphocytes stimulated with phytohaemagglutinin (PHA) over express the sense and the antisense mitochondrial chimeric RNAs.

[0103] Five ml of blood from healthy donors was collected with EDTA. The blood was diluted with one volume of 0.9% NaCl and the mixture was applied on 5 ml of Histopaque-1077 (Sigma) in a centrifuge tube. The tubes were centrifuged at 800 x g for 20 minutes at room temperature. The white cells at the interphase were collected, diluted with 2 volumes of 0.9% NaCl and centrifuged at 250 x g for 10 minutes at room temperature. The collected cells were suspended and washed twice with RPMI 1640 medium supplemented with 200 mM glutamine, 10mM non-essential amino acids, penicillin and streptomycin devoid of calf fetal serum. The final sediment was re-suspended in the same medium with 10% calf fetal serum and the number of human lymphocytes per ml was determined by counting under the microscope in a Neubauer chamber.

[0104] Human lymphocytes were cultured in 96-wells microtiter plates with the RPMI 1640 medium supplemented as described plus 10% calf fetal serum at 37°C and with 5% CO_2. About 30,000 lymphocytes per well were cultured with or without 10 ug per ml of the mitogen PHA, which induce cell proliferation (Yu et al., J. Biol. Chem., 266:7588-7595, 1991). After 48 to 72 hours of treatment with PHA, the cells are actively engaged in DNA synthesis as demonstrated by the incorporation of H3-thymidine or BrdU (Yu et al., J. Biol. Chem., 266:7588-7595, 1991). Also, 48 hours after stimulation with PHA, the lymphocytes overexpressed other markers of cell proliferation such as the proliferating cell nuclear antigen or PCNA and Ki-67 (Bantis et al., Cytopathology, 15:25-31, 2004) (Fig. 7). The resting or control lymphocytes did not express these antigens (Fig. 7).

[0105] To determine if the stimulated lymphocytes expressed the sense mitochondrial chimeric RNAs, the cells were subjected to *in situ* hybridization with oligonucleotide probes labeled with digoxigenin and targeted to the sense mitochondrial chimeric RNA. The *in situ* hybridization protocol employed was described in Example 5. A strong hybridization signal was obtained indicating overexpression of this transcript (Fig. 7). The hybridization signal was similar in intensity to that observed on tumor cells or other normal proliferating cells (compare Fig. 7 with Fig. 4A and B. Fig. 5A and B). No hybridization signal was observed on the control lymphocytes incubated without PHA (Fig. 7).

[0106] When the *in situ* hybridization was carried out with sense oligonucleotide probes labeled with digoxigenin and targeted to the antisense mitochondrial chimeric RNA, an equally strong hybridization signal was obtained (Fig. 7). Several controls were carried out to discard the possibility that the hybridization signal was due to artifacts. The hybridization signal disappears if the *in situ* hybridization is carried out with the sense labeled probe together with excess (50 to 100 times) of the same sense probe but unlabeled with digoxigenin. If prior to the hybridization the samples are incubated with ribonuclease A overnight, the hybridization signal disappears. Also, no hybridization signal is observed if the hybridization is carried out with sense probes with 4 mismatches. In contrast, *in situ* hybridization of non-stimulated lymphocyte showed no hybridization signal (Fig. 7). In conclusion, normal human lymphocytes stimulated to proliferate overexpress both, the sense mitochondrial chimeric RNA and the antisense mitochondrial chimeric RNA. These transcripts are not expressed in resting cells.

EXAMPLE 9.

The sense mitochondrial chimeric RNA exhibits different localizations in normal and tumor cells.

[0107] The *in situ* hybridizations reported in Examples 5 and 6, indicated that in several tumor cell lines as well as in tumor cells of human biopsies, the sense mtochondrial chimeric RNA is localized preferentially in the cytoplasm. However, in some tumor biopsies a clear localization of the transcripts in the nucleus was also found (FIG. 4A, B).

[0108] A surprising finding was the localization of the sense mitochondrial chimeric RNA in the nucleolus. *In situ* hybridization carried out as reported in Example 5, revealed positive hybridization signal in the nucleolus of HeLa and SiHa cells (FIG. 8. The hybridization signal was stronger in the nucleolus of HFK transformed with HPV 16 (FIG. 8). The nucleolar localization has been also found in tumor cells from breast tumors and rhabdomiosarcoma (FIG. 8).

[0109] Co-localization studies indicated that the sense mitochondrial chimeric RNA localized in the cytoplasm is outside the mitochondria and associated to late endosomes/lysosomes. If co-localization studies are carried out with markers of mitochondria such as Mitotrack (Molecular Probes), or antibodies anti-cytochrome c (Promega) or anti-Endonuclease G (Chemicon), the *in situ* hybridization showed a poor co-localization. However, a perfect co-localization was found between the hybridization signal with the immunocytochemistry of late endosemes/lysosomes markers such as Lysotrack (Molecular Probes), or antibodies anti-Lamp-2 (BD Pharmigen) or anti-cathepsin D (Zymed).

[0110] HeLa cells were subjected to *in situ* hybridization with oligonucleotide probes labeled with digoxigenin as described in Example 5. After post-hybridization and the washing procedures, the cells were incubated with an anti-digoxigenin antibody labeled with rhodamine (Roche) and an anti- Lamp-2 antibody labeled with fluorescein (BD Pharmin-

gen). After incubation at room temperature for 3 hours in the dark, the slides were washed, mounted and analyzed with a Zeiss confocal microscope. A clear co-localization of the hybridization signal with the localization of Lamp-2 was obtained. Similar co-localization results of the hybridization signal were obtained when Lysotrack or anti-cathepsin D antibodies were used as markers of the lysosomal fraction. As far as we know, this is the first report showing that a RNA (specially a mitochondrial transcript) is associated to the lysosomes of the cell. Determination of the localization of the sense mitochondrial chimeric RNA in tumor cells may have important prognostic value for patients with cancer. In general, in normal proliferating cells, the sense and the antisense mitochondrial chimeric RNAs are mainly localized in the nucleus.

EXAMPLE 10.

Treatment of tumor cells in vitro with antisense oligonucleotides targeted to the antisense mitochondrial chimeric RNA induces cell death.

[0111] HL-60 cells were cultured under the optimal conditions recommended by ATCC. About 30,000 cells were cultured in 96-well microtiter plates. Oligonucleotides (2 uM) targeted to the sense or to the antisense mitochondrial chimeric RNA were added. To enhance the permeability of the cells, the oligonucleotides were added in a mixture with lipofectamin or oligofectamin (Invitrogen) or with polyethylenimide (PEI) (Exgen TM500, Fermentas). PEI was preferred because is practically non-toxic to the cells. The cells were incubated with the oligonucleotides for 6 hours and the percentage of cell survival was determined by permeability to trypan blue. After 6 hours incubation with the oligonucleotides an important percentage of the cells died. However, oligonucleotides targeted to the antisense mitochondrial chimeric RNA were more effective to induce cell death (about 90% versus 15% of cell death). On the other hand, no apoptosis was induced when the cells were treated with oligonucleotides targeted to the sense or antisense 12S mitochondrial RNA or the mRNA of ND1 subunit or with scrambled oligonucleotides or oligonucleotides with four mismatches, all of which were used as controls. The oligonucleotides used in these studies contain phosphorothioate linkage in the first 5 nucleotides at the 5' end and the last five nucleotides at the 3' end. On the average, the 10 central nucleotides contain phosphodiester bonds.

[0112] To establish if the treatment of the cells with these oligonucleotides induces DNA fragmentation, HL-60 cells were incubated under the same conditions described before with oligonucleotides for 6 hours. About 30,000 HL-60 cells were cultured in 200 ul of IDMEM plus 10% calf fetal serum in 96-wells microtiter plate together with 1 uM of oligonucleotides targeted to the sense mitochondrial chimeric RNA or targeted to the antisense mitochondrial chimeric RNA. The chemistry of the oligonucleotides added in mixture with PEI was the same described in the previous section. After an incubation of 6 hours with the oligonucleotides the cells were assayed for DNA fragmentation using the TUNEL assay (DeadEnd Colorimetric TUNEL System, Promega). As shown in Table 2, about 96% of the cells showed DNA fragmentation after treatment with the oligonucleotide targeted to the antisense mitochondrial chimeric RNA. A similar rate of DNA fragmentation was obtained with the drug staurosporine. Scrambled oligonucleotides or oligonucleotides with mismatches showed no effect. In contrast, only about 20% of the cells died when treated with oligonucleotide targeted to the sense mitochondrial chimeric RNA (Table 2). As shown previously, tumor cells down regulate the expression of the antisense mitochondrial chimeric RNA and consequently these cells carry a low number of copies of this transcript. Therefore, cell death is more efficiently induced with oligonucleotides targeted to the antisense mitochondrial chimeric RNA. These results strongly suggest that the low number of copies of the antisense mitochondrial chimeric RNA in tumor cells constitute a target for therapy.

Table 2.

Oligonucleotides complementary to the antisense mitochondrial chimeric RNA induce apoptosis in HL-60 cells.

Treatment	Percent of apoptotic cells Assayed by TUNEL
Control	3.0 %
Oligonucleotides complementary to the antisense chimeric RNA	96.7 %
Mismatch oligonucleotides	4.0 %
Scrambled oligonucleotides	3.5 %
Oligonucleotides complementary to the sense chimeric RNA	26.7 %
Staurosporine	98.4 %
Oligonucleotides complementary to the sense 12 S mitochondrial RNA	3.7 %
Oligonucleotides complementary to the antisense 12 S mitochondrial RNA	4,1 %

[0113] In another study, we determined if the treatment of the cells with oligonucleotides targeted to the antisense mitochondrial chimeric RNA induced caspases activation. Caspases are proteolytic enzymes, actively involved in programmed cell death or apoptosis. HL-60 were incubated with oligonucleotides targeted to the antisense mitochondrial chimeric RNA or with staurosporine for 6 hours under the culturing conditions described before. Then, VAD-fmk (CaspaCe FITC-VAD-FMK, Promega) conjugated with fluorescein was added to the culture and incubated for 30 minutes at 37°C. VAD-fmok is strong inhibitor of caspases and binds to the proteases with very high affinity (Gracia-Calvo et al., J. Biol. Chem., 273:32608-32613, 1998). The cells were washed by centrifugation, mounted and observed with a fluorescence microscope. As shown in Fig. 9, HL-60 cells treated with the oligonucleotide targeted to the antisense mitochondrial chimeric RNA induced activation of caspases, at a similar level to the activation achieved with staurosporine. No activation of caspases was obtained with antisense oligonucleotides targeted to the 12S mitochondrial RNA used as control.

[0114] The cells treated with oligoncleotides targeted to the antisense mitochondrial chimeric RNA also exhibit other changes that are congruent with apoptosis. Electron microscopy analysis showed nuclear fragmentation and chromatin condensation. Nuclear fragmentation was also demonstrated by staining of the nuclei with DAPI. After treatment with these oligonucleotides targeted to the antisense mitochondrial chimeric RNA, the cells undergo nuclear fragmentation as revealed by DAPI staining (Fig. 9E and 9F).

EXAMPLE 11.

Other tumor cells also undergo cell death when treated with oligonucleotides complementary to the antisense mitochondrial chimeric RNA.

[0115] Other tumor cells were treated with oligonucleotides complementary to the antisense mitochondrial chimeric RNA according to the protocol described in Example 10. The cells were incubated in their optimal condition according to the recommendation of ATCC, and 2 uM oligonucleotide was added at the initial period of the experiment together with PEI. Six hours later a second addition of the oligonucleotide was carried out at the same concentration and the effect was determined 15 hours after the initiation of the experiment. Cell death was determined by DAP1 staining and counting the number of cells with fragmented nuclei. As shown in Table 3, over 70% of the cells treated with oligonucleotides undergo apoptosis. It is important to note that melanoma cells, lymphoma cells and the breast carcinoma cells MCF/7, known to be quite resistant to drug treatment, undergo apoptosis at a very high rate (Table 3)

Table 3.

Induction of apoptosis in tumor cell lines by treatment with oligonucleotides complementary to the antisense mitochondrial chimeric RNA.

Cells	Percent of Apoptotic Cells* (DAPI staining)
MCF/7	89 %±9
Melanome 4285	86 %±7
Hep G2	93 %±3
Hela	91 %±5
DU145	89 %±6
Lymphoma cells Devemelle	87 %±5
Caco-2	64 %±7

* Treatment was for 15 h and 2 uM oligonucleotides. Apoptosis in cells treated with scrambled or mismatch oligonucleotides, or without oligonucleotides varies between 3 to 10%.

[0116] To determine if there are regions in the transcript that are more efficient targets for the oligonucleotides in inducing apoptosis the following experiments were carried out. The induction of apoptosis was studied in Hela, HL-60 and MCF/7 cells with antisense oligonucleotides of about 20 nucleotides, targeted about every 30 nucleotides starting from the 5' end of the antisense mitochondrial chimeric RNA. At time zero, 1 uM oligonucleotides were added together with PEI and this treatment was repeated 6 hours later. Fifteen hours after the beginning of the treatment, the percentage of cells undergoing apoptosis was determined by staining with DAPI and counting the cells with fragmented nuclei. Although most of the oligonucleotides induced a variable degree of apoptosis, the single stranded region of the antisense mitochondrial chimeric RNA was a better target to induce cell death. The oligonucleotides targeted to the putative double stranded or loop structure of the antisense mitochondrial chimeric RNAs were less effective.

[0117] Apoptosis can also be determined by trypan blue staining, propidium iodide staining, anexine immunochemistry. In these techniques, the cells can be analyzed by fluorescent microscopy or by flow cytometry. DNA fragmentation can be measured by TUNEL or by electrophoresis to reveal the ladder of DNA. Western blot analysis can also be used to

determine the processing of proteins such as caspases, poly(ADP-Rib) synthase, etc.

EXAMPLE 12.

Treatment of normal proliferating or resting cells with oligonucleotides complementary to the antisense mitochondrial chimeric RNA are refractory to apoptosis.

[0118] As described before, normal proliferating cells overexpress the sense mitochondrial chimeric RNA as well as the antisense mitochondrial chimeric RNA. Resting cells, on the other hand, do not express either of these transcripts. Therefore, it was important to determine if oligonucleotides complementary to the antisense mitochondrial chimeric RNA induce cell death in normal cells.

[0119] Human lymphocytes were stimulated with 10 ug per ml of PHA for 48 hours as described in Example 8. In parallel, control lymphocytes were incubated also for 48 hours but without PHA. At 48 hours of culture, 15 uM of oligonucleotide mixed with PEI (see Example 10) was added to the stimulated and control lymphocytes and further incubated with 15 hours. The concentration of the oligonucleotide was 10 fold higher than the concentration used in previous experiments (1-2 uM). Other samples of stimulated or control lymphocytes were treated with 0.4 uM staurosporine for the same period of time. At the end of the experiment, cell death was measured by either trypan blue staining or DAPI staining. As shown in Fig. 10, control lymphocytes or lymphocytes stimulated with PHA incubated for 15 hours without oligonucleotide showed a similar level of spontaneous apoptosis that varied between 7 to 10% in different experiments. A similar result was obtained with a lower (1-2 uM) concentration of oligonucleotide. Also, control and stimulated lymphocytes incubated with 15 uM antisense oligonucleotide for 15 hours showed a similar low level of apoptosis (around 10%) (Fig. 10). In contrast, control lymphocytes or lymphocytes stimulated with PHA and also incubated with staurosporine for 15 hours showed that over 80% of the cells undergo apoptosis (Fig. 10). This is a very important result because it shows that normal resting cells or normal proliferating cells such as human lymphocytes are refractory to induction of apoptosis by the oligonucleotides complementary to the antisense mitochondrial chimeric RNA. In other words, induction of apoptosis in tumor cells by interfering with the antisense mitochondrial chimeric RNA is a selective therapeutic approach for cancer.

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Claims

1. A pharmaceutical composition, wherein the pharmaceutical composition comprises one or more antisense oligonucleotides of 10-50 nucleobases in length which are complementary to a human mitochondrial chimeric RNA molecule comprising a sense or antisense 16S mitochondrial RNA covalently linked at its 5' end to the 3' end of a polynucleotide with an inverted repeat sequence, to be able to hybridize with that human mitochondrial chimeric RNA molecule to form a stable duplex, further wherein one or more of the oligonucleotides comprise at least one alternate internucleoside linkage.

2. The pharmaceutical composition of claim 1, wherein said one or more oligonucleotides are selected from the group consisting of SEQ ID NOs: 9-196.

3. The pharmaceutical composition of claim 1 or claim 2, wherein the human mitochondrial chimeric molecule comprises an antisense 16S mitochondrial RNA covalently linked at its 5' end to the 3' end of a polynucleotide with an inverted repeat sequence.

4. The pharmaceutical composition of claim 3, wherein the human mitochondrial chimeric molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 4, 5, and 6.

5. The pharmaceutical composition of claim 1 or claim 2, wherein the human mitochondrial chimeric molecule comprises a sense 16S mitochondrial RNA covalently linked at its 5' end to the 3' end of a polynucleotide with an inverted repeat sequence.

6. The pharmaceutical composition of claim 5, wherein the human mitochondrial chimeric molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1,2, and 3.

7. The pharmaceutical composition of claim 1, wherein the alternate internucleoside linkage is a phosphorothioate internucleosidic linkage.

8. The pharmaceutical composition of claim 7, wherein said one or more oligonucleotides further comprises one or more 2-o-(2-methoxy) ethyl modified 5' end oligonucleotides and one or more 2-o-(2-methoxy) ethyl modified 3' end oligonucleotides.

9. The pharmaceutical composition of claim 1, wherein said one or more oligonucleotides comprises one or more locked oligonucleotides.

10. The pharmaceutical composition of claim 1, wherein said one or more oligonucleotides comprises one or more peptidic nucleic acids.

11. The pharmaceutical composition of any of claims 1-10, wherein the composition is formulated for topical, oral, parenteral, or rectal administration.

12. The pharmaceutical composition of any one of claims 1-10, for use in the treatment of cancer or precancer.

Patentansprüche

1. Pharmazeutische Zusammensetzung, wobei die pharmazeutische Zusammensetzung ein oder mehrere Antisense-Oligonucleotid(e) mit einer Länge von 10-50 Nucleobasen umfasst, die zu einem menschlichen mitochondrialen chimären RNA-Molekül komplementär sind, umfassend eine Sense- oder Antisense-16S-mitochondriale RNA in kovalenter Bindung an deren 5'-Ende an das 3'-Ende eines Polynucleotids mit einer invertierten Repeat-Sequenz zur Befähigung zur Hybridisierung mit diesem humanen mitochondrialen chimären RNA-Molekül unter Bildung einer stabilen Duplex, wobei weiterhin eines oder mehrere der Oligonucleotide mindestens eine alternative Internucleosidbindung umfasst.

2. Pharmazeutische Zusammensetzung nach Anspruch 1, wobei das eine oder mehrere Oligonucleotid(e) aus der Gruppe, bestehend aus SEQ ID NOs: 9-196 ausgewählt ist bzw. sind.

3. Pharmazeutische Zusammensetzung nach Anspruch 1 oder 2, wobei das humane mitochondriale chimäre Molekül eine 16S mitochondriale Antisense-RNA in kovalenter Bindung an deren 5'-Ende an das 3'-Ende eines Polynucleotids mit einer invertierten Repeat-Sequenz umfasst.

4. Pharmazeutische Zusammensetzung nach Anspruch 3, wobei das humane mitochondriale chimäre Molekül eine Nucleotidsequenz ausgewählt aus der Gruppe, bestehend aus SEQ ID NOs: 4, 5 und 6 umfasst.

5. Pharmazeutische Zusammensetzung nach Anspruch 1 oder 2, wobei das humane mitochondriale chimäre Molekül eine 16S mitochondriale Sense-RNA in kovalenter Bindung an deren 5'-Ende an das 3'-Ende eines Polynucleotids mit einer invertierten Repeat-Sequenz umfasst.

6. Pharmazeutische Zusammensetzung nach Anspruch 5, wobei das humane mitochondriale chimäre Molekül eine Nucleotidsequenz ausgewählt aus der Gruppe, bestehend aus SEQ ID NOs: 1, 2 und 3 umfasst.

7. Pharmazeutische Zusammensetzung nach Anspruch 1, wobei die alternative Internucleosidbindung eine Phosphorothioat-Internucleosidbindung ist.

8. Pharmazeutische Zusammensetzung nach Anspruch 7, wobei das eine oder mehrere Oligonucleotid(e) ein oder mehrere am 5'-Ende 2-o-(2-Methoxy)ethyl modifizierte Oligonucleotid(e) und ein oder mehrere am 3'-Ende 2-o-(2-Methoxy)ethyl modifizierte Oligonucleotid(e) umfasst bzw. umfassen.

9. Pharmazeutische Zusammensetzung nach Anspruch 1, wobei das eine oder mehrere Oligonucleotid(e) ein oder mehrere blockierte Oligonucleotid(e) umfasst bzw. umfassen.

10. Pharmazeutische Zusammensetzung nach Anspruch 1, wobei das eine oder mehrere Oligonucleotid(e) ein oder mehrere peptidische Nucleinsäure(n) umfasst bzw. umfassen.

11. Pharmazeutische Zusammensetzung nach einem der Ansprüche 1-10, wobei die Zusammensetzung für topische, orale, parenterale oder rektale Verabreichung formuliert ist.

12. Pharmazeutische Zusammensetzung nach einem der Ansprüche 1-10 zur Verwendung zur Behandlung von Krebs oder Prä-Krebs.

Revendications

1. Composition pharmaceutique, la composition pharmaceutique comprenant un ou plusieurs oligonucléotide(s) antisens de 10-50 bases nucléiques de longueur, qui est(sont) complémentaire(s) d'une molécule d'ARN chimérique mitochondriale humaine comprenant un ARN mitochondrial 16S sens ou antisens lié par covalence par son extrémité 5' à l'extrémité 3' d'un polynucléotide ayant une séquence répétée inversée, pour pouvoir s'hybrider avec cette molécule d'ARN chimérique mitochondriale humaine pour former un duplex stable, en outre dans laquelle un ou plusieurs des oligonucléotides comprend(comprennent) au moins une autre liaison internucléotidique.

2. Composition pharmaceutique selon la revendication 1, dans laquelle le(s)dit(s) un ou plusieurs oligonucléotide(s) est(sont) choisi(s) dans l'ensemble constitué par les Séquences N° 9 à 196.

3. Composition pharmaceutique selon la revendication 1 ou la revendication 2, dans laquelle la molécule chimérique mitochondriale humaine comprend un ARN mitochondrial 16S antisens lié par covalence par son extrémité 5' à l'extrémité 3' d'un polynucléotide ayant une séquence répétée inversée.

4. Composition pharmaceutique selon la revendication 3, dans laquelle la molécule chimérique mitochondriale humaine comprend une séquence de nucléotides choisie dans l'ensemble constitué par les Séquences N° 4, 5 et 6.

5. Composition pharmaceutique selon la revendication 1 ou la revendication 2, dans laquelle la molécule chimérique mitochondriale humaine comprend un ARN mitochondrial 16S sens lié par covalence par son extrémité 5' à l'extrémité 3' d'un polynucléotide ayant une séquence répétée inversée.

6. Composition pharmaceutique selon la revendication 5, dans laquelle la molécule chimérique mitochondriale humaine comprend une séquence de nucléotides choisie dans l'ensemble constitué par les Séquences N° 1, 2 et 3.

7. Composition pharmaceutique selon la revendication 1, dans laquelle l'autre liaison internucléotidique est une liaison internucléotidique phosphorothioate.

8. Composition pharmaceutique selon la revendication 7, dans laquelle le(s)dit(s) un ou plusieurs oligonucléotide(s) comprend(comprennent) en outre un ou plusieurs oligonucléotide(s) à extrémité 5' modifiée avec un groupe 2-o-(2-méthoxy)-éthyle et un ou plusieurs oligonucléotide(s) à extrémité 3' modifiée avec un groupe 2-o-(2-méthoxy)éthyle.

9. Composition pharmaceutique selon la revendication 1, dans laquelle le(s)dit(s) un ou plusieurs oligonucléotide(s) comprend(comprennent) un ou plusieurs oligonucléotide(s) bloqué(s).

10. Composition pharmaceutique selon la revendication 1, dans laquelle le(s)dit(s) un ou plusieurs oligonucléotide(s) comprend(comprennent) un ou plusieurs acide(s) nucléique(s) peptidique(s).

11. Composition pharmaceutique selon l'une quelconque des revendications 1 à 10, la composition étant formulée pour administration topique, orale, parentérale ou rectale.

12. Composition pharmaceutique selon l'une quelconque des revendications 1 à 10, pour utilisation dans le traitement d'un cancer ou pré-cancer.

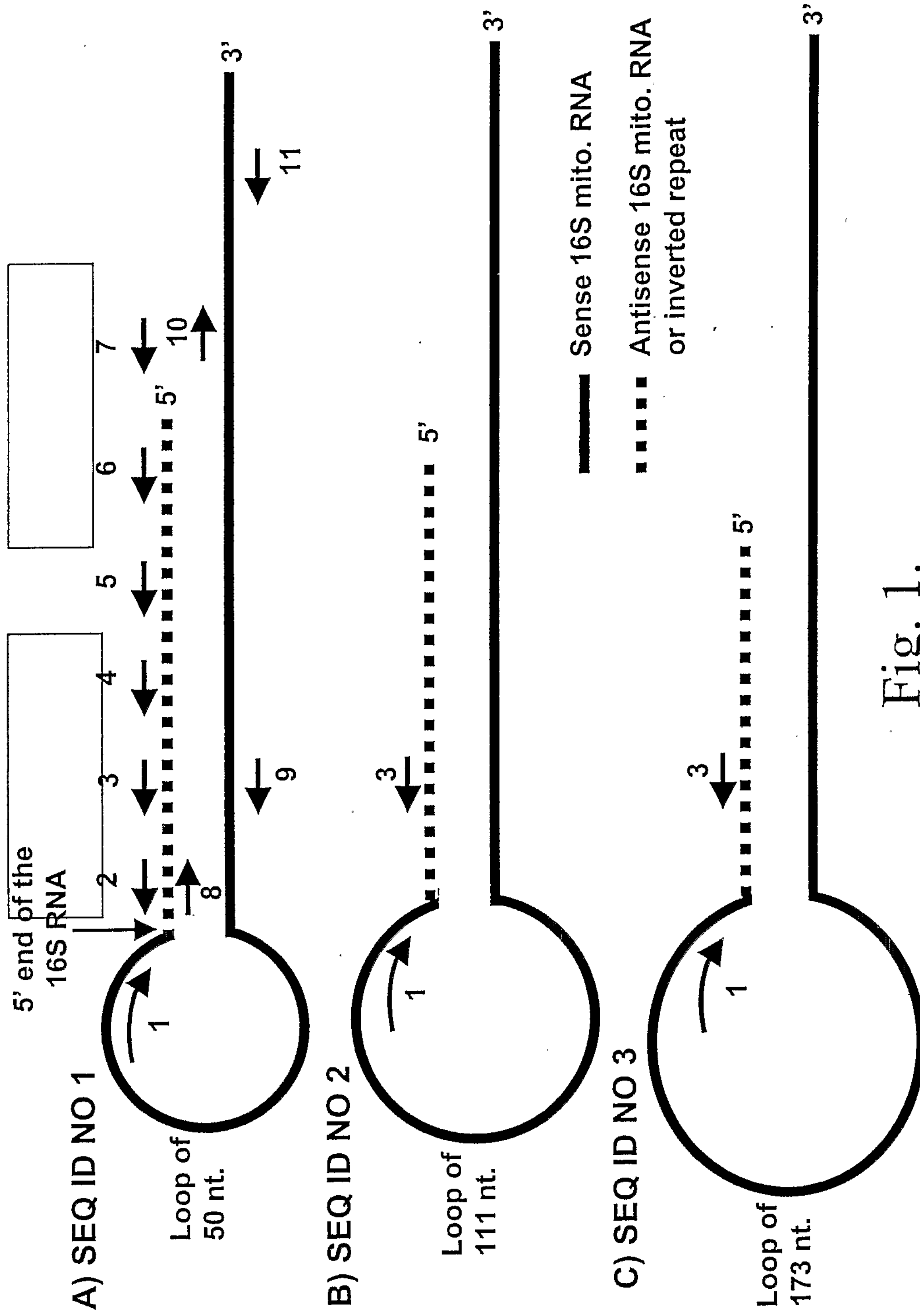

Fig. 1.

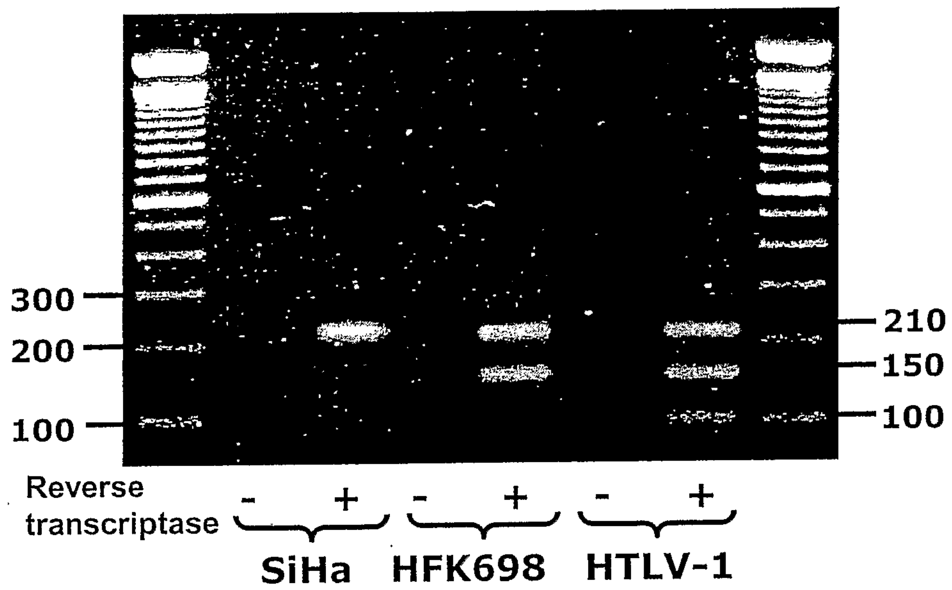

FIG. 2.

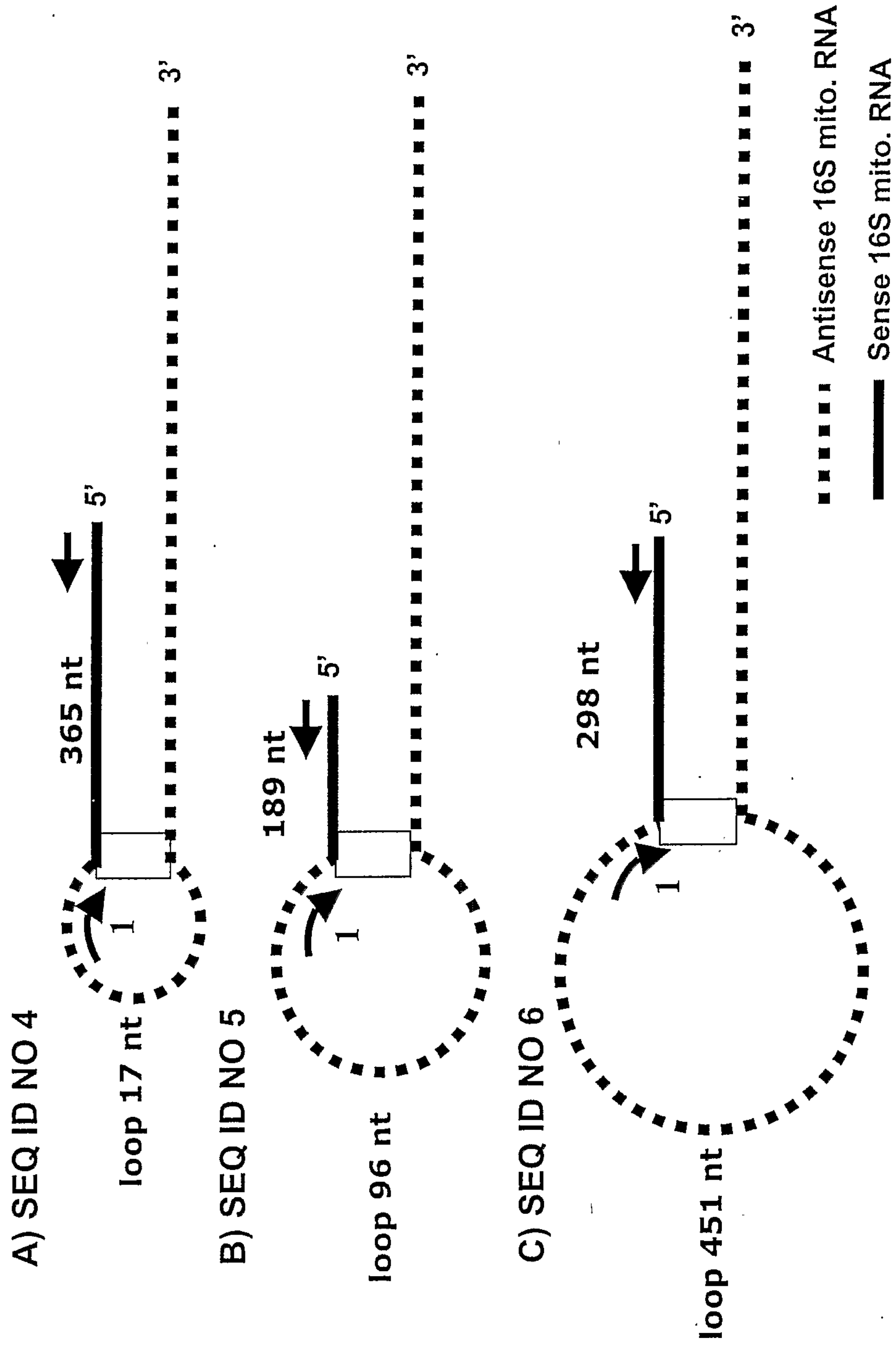

Fig. 3

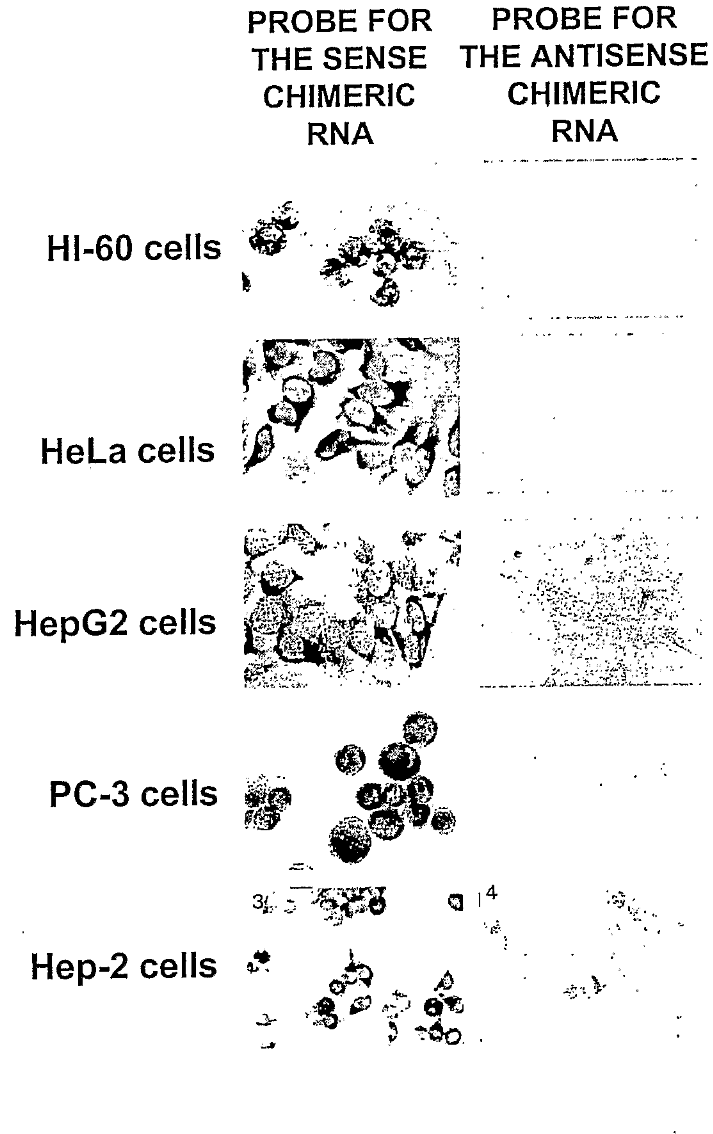

Fig. 4A

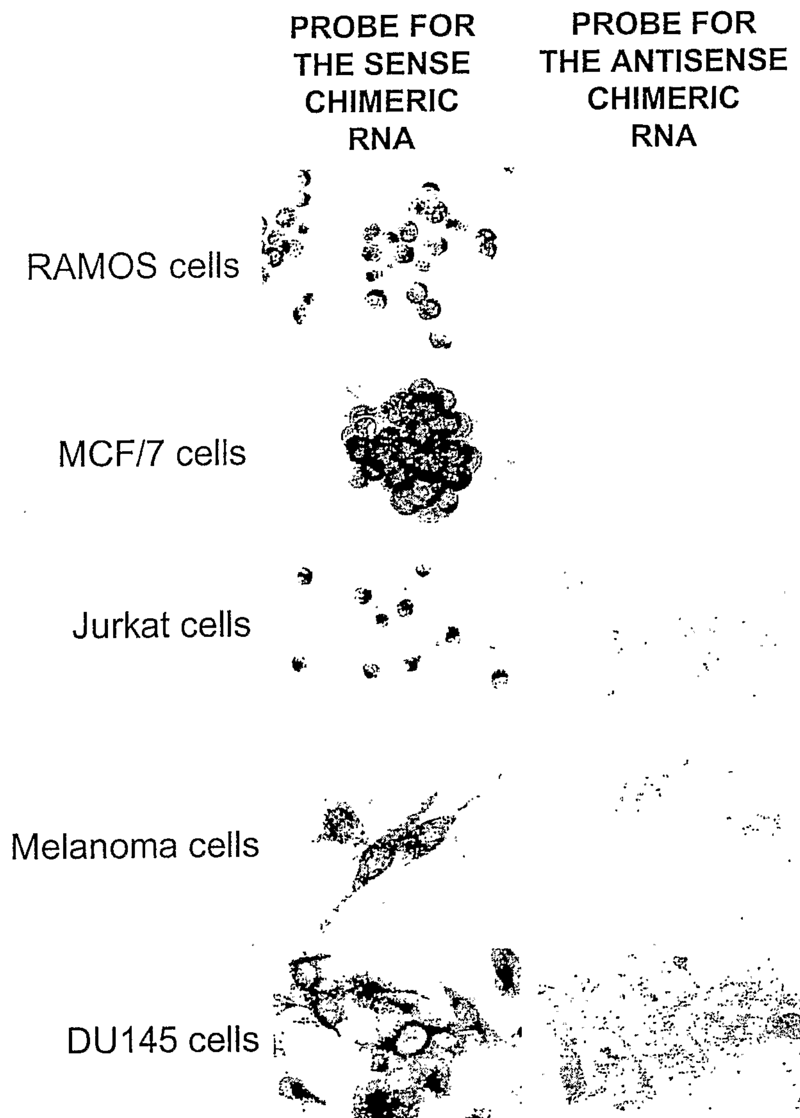

Fig. 4B

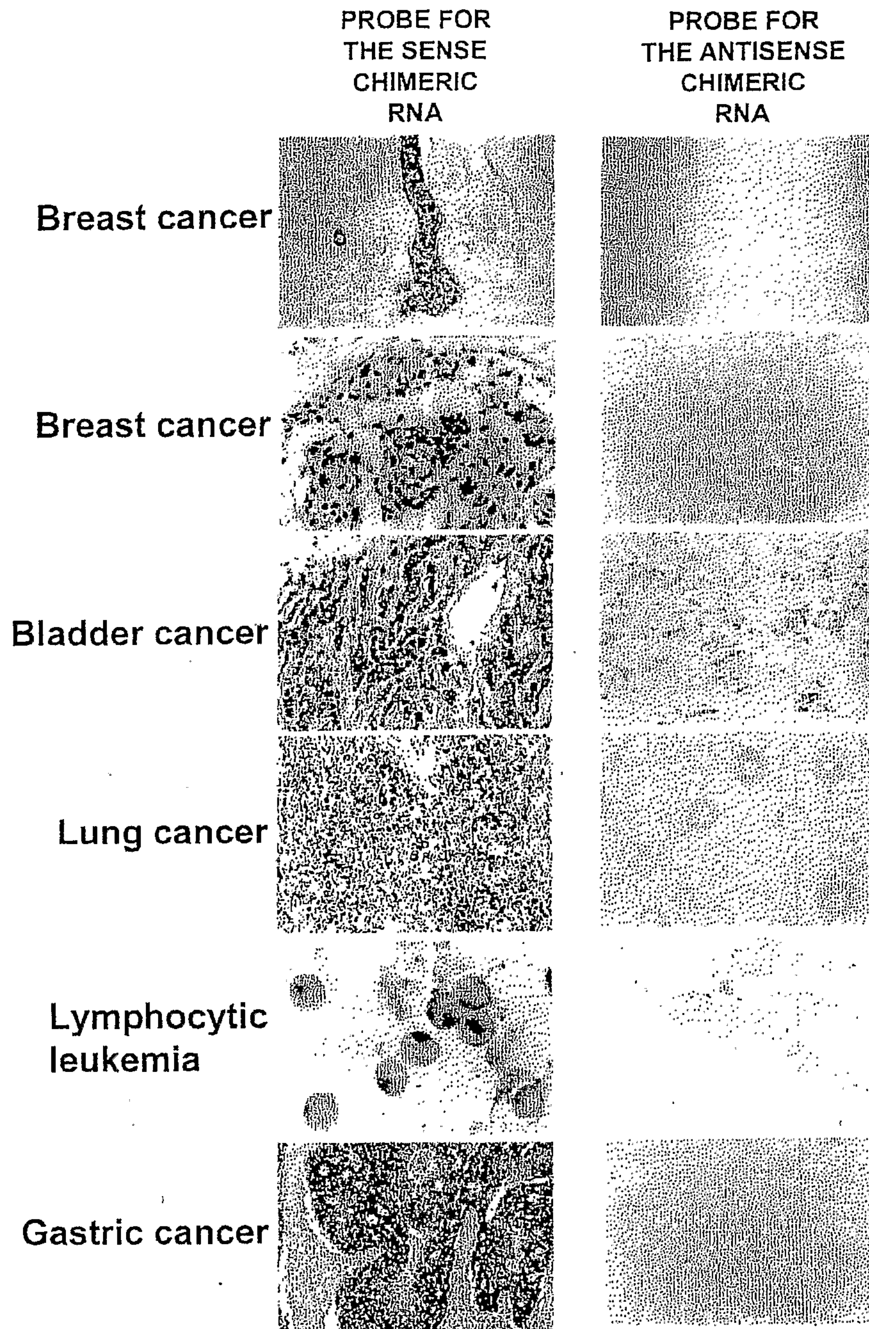

Fig. 5A

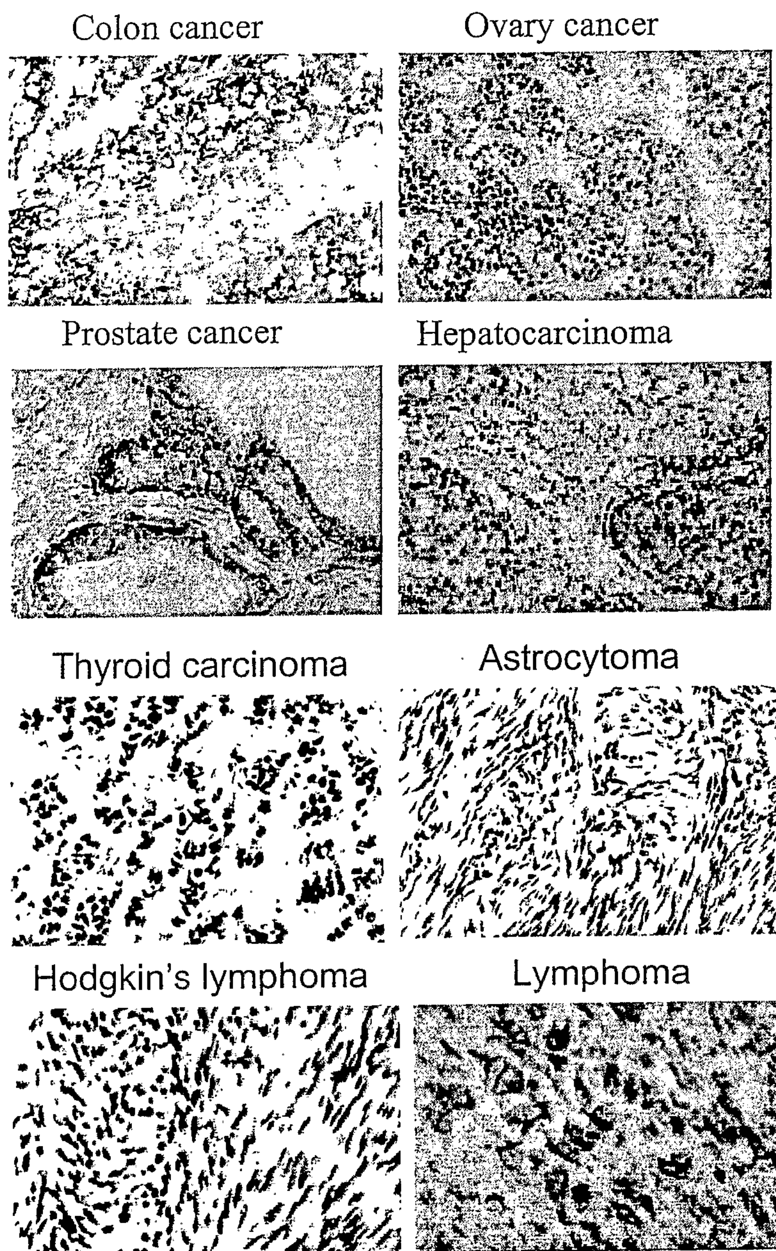

FIG. 5B

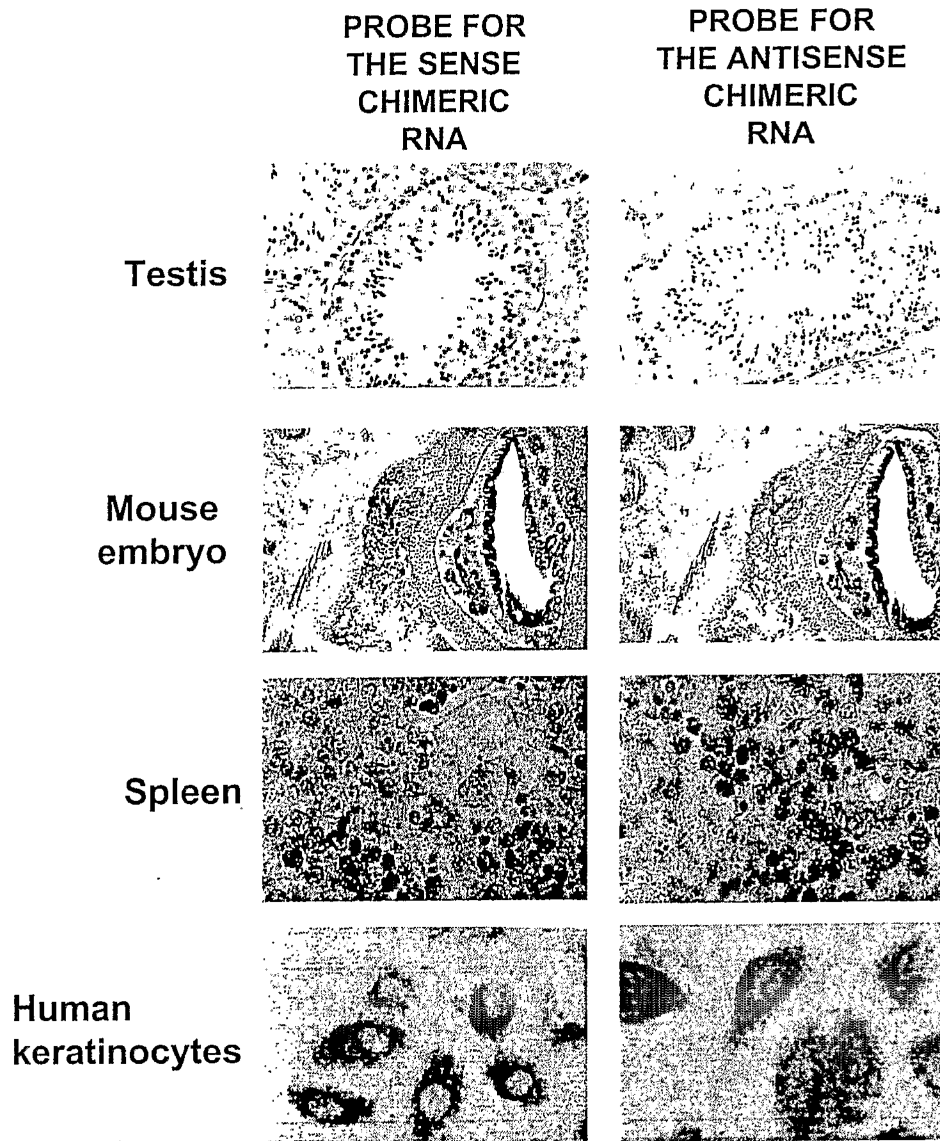

Fig. 6

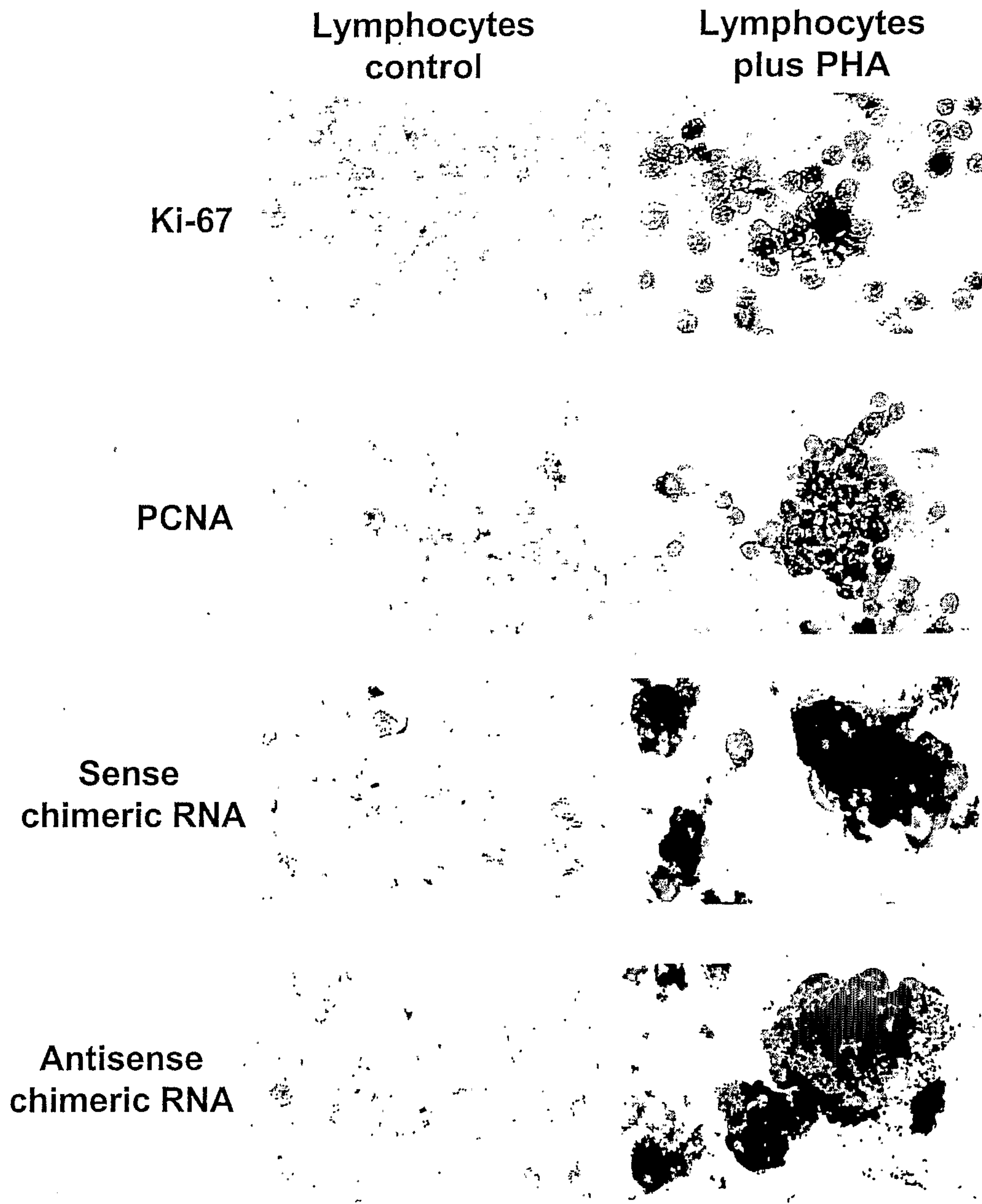

Fig. 7

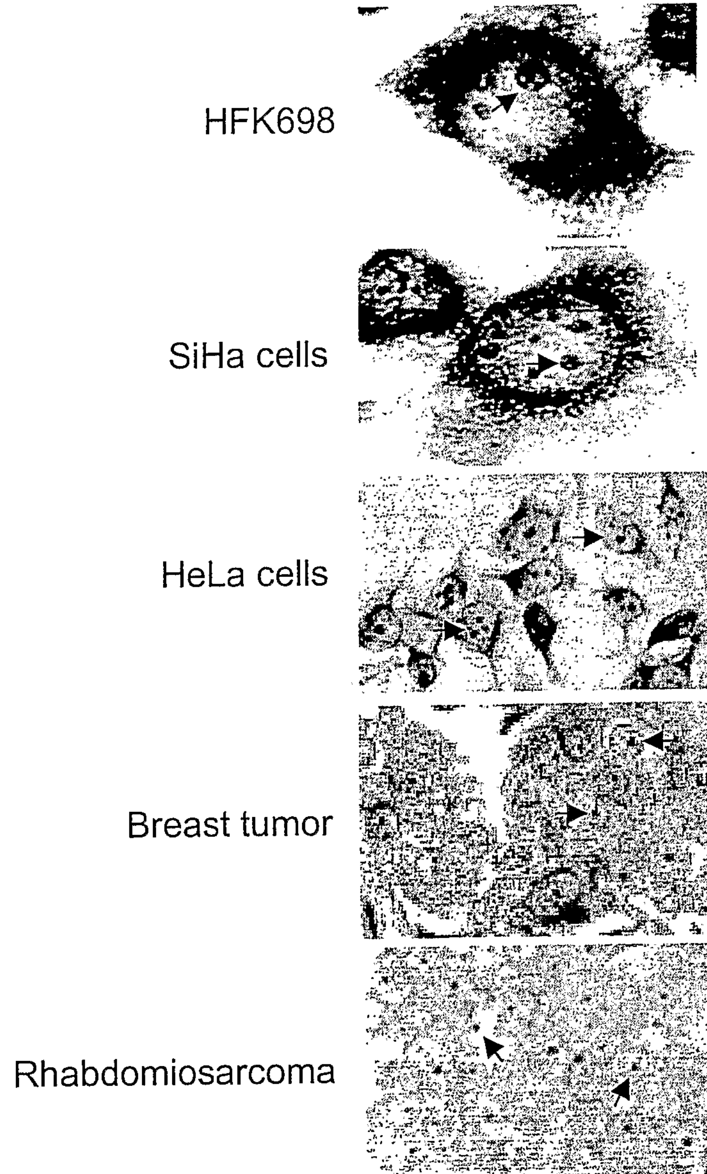

FIG. 8.

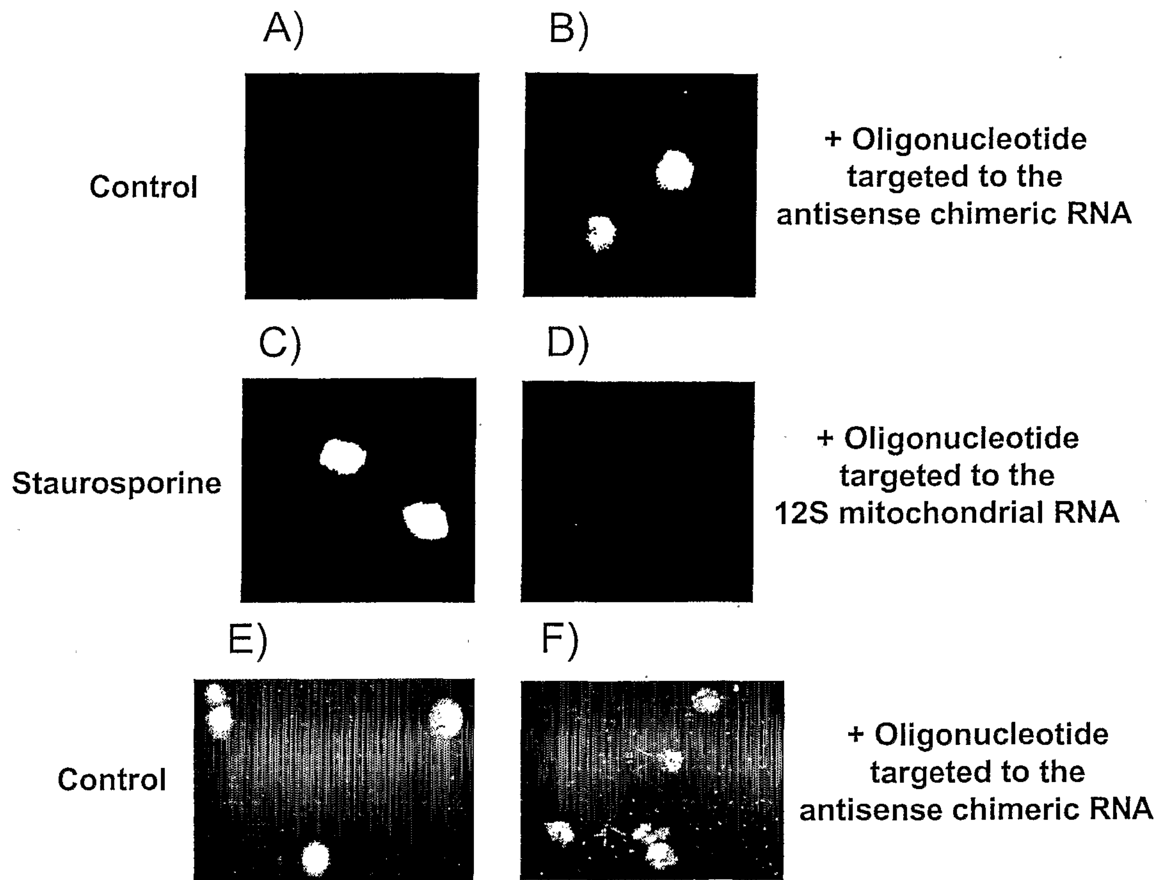

Fig. 9

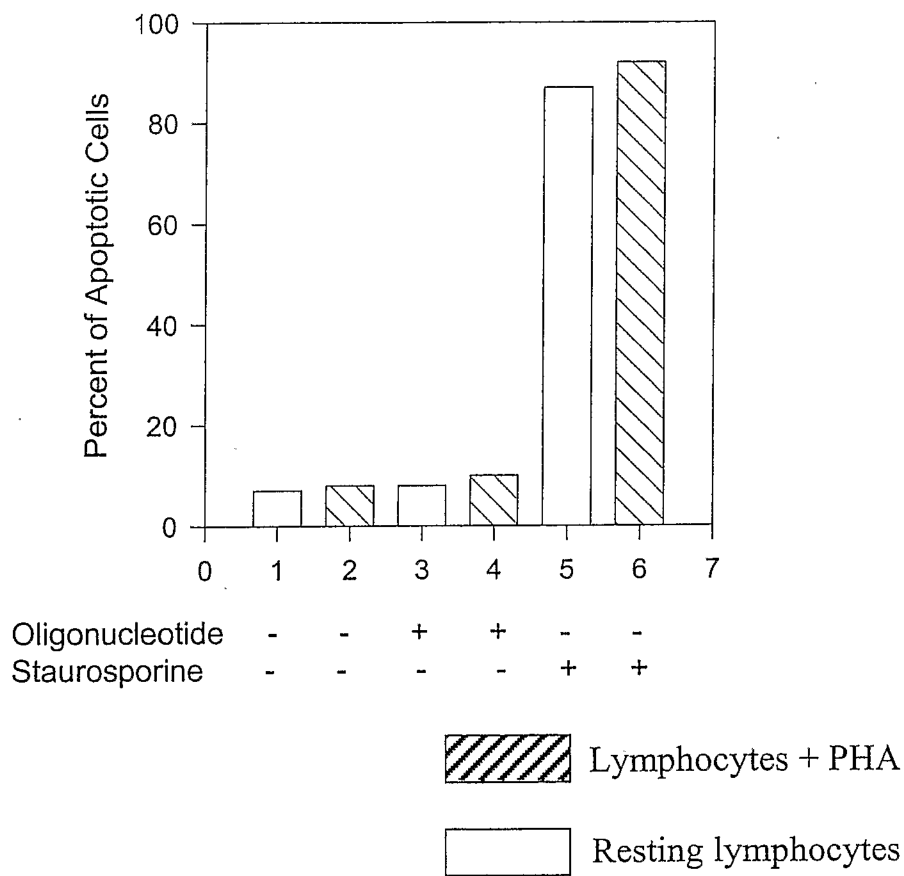

Fig. 10.

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SBGK Szabadalmi Ügyvivői Iroda

Vegyületek és oligonukleotidok rák, és rák-megelőző állapot diagnosztizálására és kezelésére

SZABADALMI IGÉNYPONTOK

1. Gyógyászati készítmény, amely tartalmaz egy vagy több, 10-50 nukleobázis hosszúságú antiszensz oligonukleotidot, amelyek komplementerek egy humán mitokondriális kiméra RNS molekulával, amely egy értelmes vagy antiszensz 16S mitokondriális RNS-t tartalmaz, amely 5' végén kovalens kötéssel kapcsolódik egy inverz ismétlődő szekvenciával rendelkező polinukleotid 3' végéhez, hogy képes legyen hibridizálódni a szóban forgó humán mitokondriális kiméra RNS molekulával, és stabil duplexet képez, továbbá, amelyben egy vagy több oligonukleotid legalább egy váltakozó internukleozid kötést tartalmaz.

2. Az 1. igénypont szerinti gyógyászati készítmény, amelyben a szóban forgó egy vagy több oligonukleotidot a 9-196. számú szekvenciák közül választjuk ki.

3. Az 1. vagy 2. igénypont szerinti gyógyászati készítmény, amelyben a humán mitokondriális kiméra molekula tartalmaz egy antiszensz 16S mitokondriális RNS-t, amely 5' végén kovalens kötéssel kapcsolódik egy inverz ismétlődő szekvenciával rendelkező polinukleotid 3' végéhez.

4. A 3. igénypont szerinti gyógyászati készítmény, amelyben a humán mitokondriális kiméra molekula tartalmaz egy nukleotid szekvenciát, amelyet a 4., 5. vagy 6. számú szekvenciák közül választhatunk ki.

5. Az 1. vagy 2. igénypont szerinti gyógyászati készítmény, amelyben a humán mitokondriális kiméra molekula tartalmaz egy értelmes 16S mitokondriális RNS-t, amely 5' végén kovalens kötéssel kapcsolódik egy inverz ismétlődő szekvenciával rendelkező polinukleotid 3' végéhez.

6. Az 5. igénypont szerinti gyógyászati készítmény, amelyben a humán mitokondriális kiméra molekula tartalmaz egy nukleotid szekvenciát, amelyet az 1., 2. vagy 3. számú szekvenciák közül választhatunk ki.

7. Az 1. igénypont szerinti gyógyászati készítmény, amelyben a váltakozó internukleozid kötés egy foszforotioát internukleozid kötés.

8. A 7. igénypont szerinti gyógyászati készítmény, amelyben a szóban forgó egy vagy több oligonukleotid tartalmaz még egy vagy több 2-o-(2-metoxi)-etil-csoporttal módosított 5' végű oligonukleotidot, és egy vagy több 2-o-(2-metoxi)-etil-csoporttal módosított 3' végű oligonukleotidot.

9. Az 1. igénypont szerinti gyógyászati készítmény, amelyben a szóban forgó egy vagy több oligonukleotid tartalmaz egy vagy több lezárt oligonukleotidot.

10. Az 1. igénypont szerinti gyógyászati készítmény, amelyben a szóban forgó egy vagy több oligonukleotid tartalmaz egy vagy több peptid-nukleinsavat.

11. Az 1-10. igénypontok bármelyike szerinti gyógyászati készítmény, ahol a készítmény topikális, orális, parenterális, vagy rektális beadáshoz van kiszerelve.

12. Az 1-10. igénypontok bármelyike szerinti gyógyászati készítmény, rák, vagy rák-megelőző állapot kezelésében történő alkalmazásra.

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