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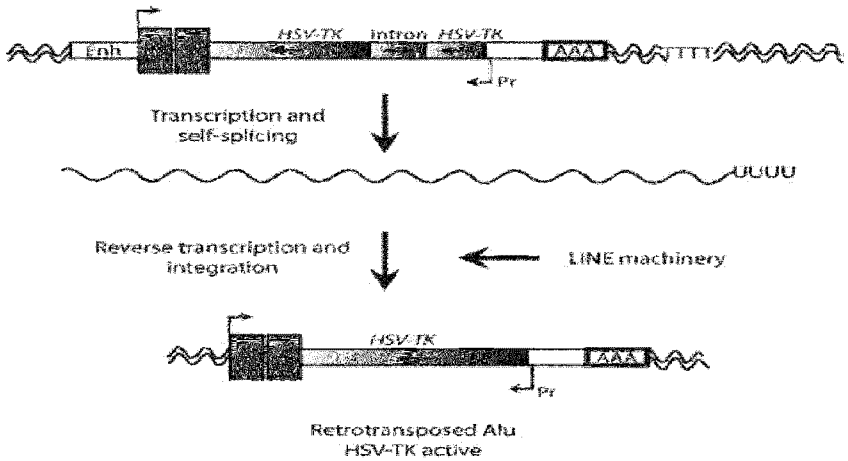


Figure 3(A)

(57) Abstract: The present invention relates to the use of Alu-retrotransposon-based vectors for gene therapy, in particular cancer gene therapy. The invention also provides pharmaceutical compositions comprising such Alu-retrotransposon-based vectors and gene therapy methods for treating a cancer patient using an Alu-retrotransposon vector, or a pharmaceutical composition thereof.

Synthetic Alu-Retrotransposon Vectors for Gene Therapy

Related Patent Application

The present application claims priority to European Patent Application number EP 14 182 697, which was filed on August 28, 2014. The European patent application is incorporated herein by reference in its entirety.

Background of the Invention

In patients with advanced solid tumors or recurrences despite surgery, chemotherapy and/or radiation can provide quality survival. Drugs currently available are not cancer-specific so functional concentration level in tumors cannot be reached without off-target toxicity level. The limitations of traditional cytotoxic chemotherapy make it necessary to explore other therapies; one of them is targeted suicide gene therapy. Gene therapy appears as a good alternative and holds great promises. Suicide gene therapy (also known as Gene Directed Enzyme/Prodruct Therapy or GDEPT) exhibits advantages over classical therapy. As only tumor cells possess the enzyme that converts the prodrug into active metabolites, the toxicity level is increased several folds inside the tumor whereas the vast majority of the host cells are unaffected. By using tumor-specific regulatory elements (such as the Survivin promoter), drugs can be activated only in tumor cells. Although suicide gene therapy for cancer treatment started more than 25 years ago, it is still in its infancy as a treatment modality. However encouraging signs of clinical benefit are emerging for some patients. According to current statistics, 1550 clinical trials of gene therapy have been reported worldwide (7% of which concerning suicide gene therapy). The most widely used suicide genes for cancer therapy are Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK) and Cytosine Deaminase (CD) from the virus Herpes simplex or the bacterium Escherichia coli, respectively or cytochrome P450.

GDEPT is reaching an exciting stage with several early stage trials completed and phase III trials initiated or well advanced. Early stage trials for brain tumors using AdV/TK and ganciclovir in combination with radiation are also recruiting patients. Trials for locally recurrent and metastatic prostate cancer have been completed and encouraging signs of safety (even with up to nine courses of vector/prodrug treatment)

and clinical benefit have emerged (Freytag *et al.*, Mol. Ther., 2007, 15(5): 1016-1023). The PNP/fludarabine system has received approval to enter in first phase I trial for prostate cancer using a non-replicating ovine AdV vector for gene delivery. A next generation AdVa vector which incorporates the AdV "death protein" for improved cell lysis for prostate cancer combined with CD fused to an improved TK gene is now entering a phase III trial for prostate cancer in conjunction with radiotherapy. Phase I trials for pancreatic cancer have been initiated with the same AdV vector in combination with chemotherapy and radiation or with a non-replicating AdV/TK vector in conjunction with surgery, chemotherapy and radiation. It is encouraging that in clinical trials carried out to date, the maximum tolerated dose of GDEPT was usually not reached and adverse events were generally mild and transient, reflecting the general safety of the approach.

Classic ways to achieve good expression of alien genes in vertebrates are based on various methods of gene construct delivery in cell culture, such as transfection by electroporation, sonoporation, needless injection, etc. The main problems with these approaches center around the low integration efficiency and unstable expression of the constructs, which can be explained by the injected DNA concatemerization preceding its integration into the genome. Retrovirus and lentivirus, carriers of choice in most gene therapy studies, present a variety of potential problems to the patient – toxicity, immune and inflammatory responses, and gene control and targeting issues. Cationic liposomes or other DNA-delivery systems could represent an interesting alternative.

The use of DNA retrotransposons holds the promise of lower immunogenicity, enhanced safety profile and reduced manufacturing costs compared to viral vectors. Retrotransposons or retroelements are naturally occurring DNA elements which are found in cells from almost all species of animals, plants and bacteria that have been examined to date. They are mobile elements that insert into new genomic locations by a mechanism that involves reverse transcription of an RNA intermediate. Retrotransposons may be grouped into at least three classes that are structurally distinct and also retrotranspose using radically different mechanisms. These three families are: exogenous retroviruses, retrovirus-like LTR retrotransposons and the non-LTR elements such as human LI elements and SINE elements (Alu). Non-LTR

retrotransposons can be subdivided further into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs).

Two DNA transposons from non-mammalian species have emerged as gene therapy tools due to their efficient transposition in humans: the reconstructed Tc1/mariner element Sleeping Beauty from salmonid fish and PiggyBac (PB) from the baculovirus genome. The transposase in trans can act on any DNA sequence that is flanked by terminal repeat sequences, which are normally found at the end of transposons. Therefore, to convert a transposon into a gene delivery tool, a dual system has to be developed consisting of an expression plasmid containing DNA transposase and a donor plasmid containing the DNA to be integrated, flanked by terminal repeats. For example, the SB transposon-based vectors have successfully been used to deliver the genes sFIt-1 (soluble vascular endothelial growth factor receptor) and statin-AE (angiostatin-endostatin fusion gene) into human glioblastoma. Such transformation decreased the tumor size and increased the proportion of animals that survived. Today, a new generation of "hyperactive" SB-based vectors is used, such as SB100X. A good example of the efficiency of such vectors come from another study in which Kang *et al.* (Cell Biol. Int., 2009, 33(4): 509-515) applied GDEPT using a PB-based vector to treat ovarian adenocarcinoma. Based on the results obtained, Kang *et al.* argue that PB is the most efficient transposon for stable genomic integration among the known mammal systems. Potential side effects of these therapies are related to secondary mutagenesis resulting from exogenous transposon hopping or activation of nearby genes.

Thus, even though progresses have been made in the field, there still remains a need in the art for new tools to deliver suicide genes specifically in cancer cells thereby attacking cancer cells in a gene therapy approach.

Summary of the Invention

The present Applicants have shown that an Alu retroelement can be used in cancer gene therapy to deliver a suicide gene selectively in cancer cells. Since the insertion of SINEs requires expression of LINE-1, which is the most commun human long interspersed nuclear element (LINE), and since LINE-1 expression is higher in cancer cells than in healthy cells, the expression/integration of the suicide gene is significantly higher in cancer cells and proportional to LINE-1 expression in the cells, thereby specifically targeting the suicide gene expression to cancer cells.

Accordingly, in one aspect, the present invention provides an expression construct or vector for use as a gene therapy agent, wherein the expression construct or vector comprises an Alu retrotransposon flanked with a gene or nucleic acid sequence. The gene or nucleic acid sequence is to be delivered to a cell that overexpresses LINE-1.

The present invention also provides an expression construct or vector for use in gene therapy for the treatment of cancer in a subject, wherein the expression construct or vector comprises an Alu retrotransposon flanked with a gene or nucleic acid sequence.

In certain embodiments, the cancer to be treated overexpresses LINE-1.

Preferably, an expression construct or vector according to the present invention further comprises suitable regulatory sequences operably linked to the Alu retrotransposon and/or to the gene or nucleic acid sequence.

In certain embodiments, the Alu retrotransposon present in an expression construct or vector of the present invention belongs to the AluY subfamily of retrotransposons, preferably to the AluYa8 subfamily or to the AluYa5 subfamily.

In certain embodiments, the Alu retrotransposon present in an expression construct or vector of the present invention has the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or has a sequence that is at least 80% homologous to SEQ ID NO: 1 or SEQ ID NO: 2. When the Alu retrotransposon has a sequence that is at least 80% homologous to SEQ ID NO: 1 or SEQ ID NO: 2, it hybridizes specifically to the sequence complementary to the sequence set forth in SEQ ID NO: 1 or in SEQ ID NO: 2, respectively under stringent hybridization conditions or moderately stringent hybridization conditions.

In certain embodiments, the gene present in the construct of the present invention is placed in the opposite transcriptional orientation to the Alu element (*i.e.*, the Alu element is flanked to the non-coding sequence of the gene) and the sequence of the gene is interrupted by a small artificial autocatalytic intron. The small autocatalytic intron may be the autocatalytic Tetrahymena (Tet) intron.

In certain embodiments, the gene present in the construct of the present invention is a suicide gene. The suicide gene may be selected from the group consisting of the thymidine kinase gene (HSV-TK) of herpes simplex virus, the cytosine deaminase (CD)

gene of *Escherichi coli*, the human carboxyl esterase gene (CE) isoform1, the hepatic cytochrome P540-2B1 gene, the nitroreductase gene, the varicella-zoster virus thymidine kinase (VZV-TK) gene, the purine nucleoside phosphorylase (PNP) gene, the beta-galatosidase gene, the linamarase gene, the horseradish peroxidase gene, the carboxypeptidase A gene and the carboxypeptidase G2 gene.

In certain embodiments, the gene present in the construct of the present invention is a therapeutic gene selected from the group consisting of genes that encode a tumor suppressor, genes that encode an inhibitor of apoptosis, genes that encode a toxin, genes that encode a cytokine and cell cycle regulatory genes.

In certain embodiments, the nucleic acid sequence present in the construct of the present invention is a RNA interfering (RNAi) agent.

In certain embodiments, the expression construct or vector according to the present invention comprises a plasmid vector or a viral vector. The viral vector may be selected from adenoviral vectors, adeno-associated viral vectors, retroviral vectors and lentiviral vectors.

In certain embodiments, the expression construct or vector is administered in nanoparticles.

In certain embodiments, the expression construct comprises an Alu retrotransposon flanked with a HSV-TK gene and has the sequence set forth in SEQ ID NO: 4 or SEQ ID NO: 5.

In a related aspect, the present invention provides a pharmaceutical composition for use in gene therapy, wherein the pharmaceutical composition comprises a therapeutically effective amount of an expression construct or vector according to the invention and at least one physiologically acceptable carrier or excipient. The gene or nucleic acid sequence present in the expression construct or vector is to be delivered to a cell that overexpresses LINE-1.

In certain embodiments, the pharmaceutical composition is used for gene therapy in the treatment of cancer. Preferably, the cancer to be treated overexpresses LINE-1.

In yet related another aspect, the present invention provides a gene therapy method for the treatment of cancer in a subject, wherein the method comprises a step of administering a therapeutically effective amount of an expression construct or vector

according to the invention, or a pharmaceutical composition thereof, to the subject. Preferably, the cancer to be treated overexpresses LINE-1.

In certain embodiments, the cancer to be treated is previously tested to determine that it overexpresses LINE-1.

In certain embodiments, the cancer to be treated is selected from the group consisting of breast cancers, bladder cancers, ovary cancers, lung cancers, stomach cancers, colon cancers, and pharynx cancers.

In another aspect, the present invention concerns the use of an expression construct or vector according to the invention, or a pharmaceutical composition thereof, for delivering a gene or nucleic acid sequence to a cell that overexpresses LINE-1. In certain embodiments, the cell that overexpresses LINE-1 is an *in vitro* cell. In other embodiments, the cell that overexpresses LINE-1 is an *in vivo* cell.

In a related aspect, the present invention provides a method for delivering a gene or nucleic acid sequence to a cell that overexpresses LINE-1, wherein the method comprises introducing an expression construct or vector according to the invention into the cell.

In certain embodiments the step of introducing is achieved *via* transformation, transduction, transfection or infection.

In other embodiments, the step of introducing is achieved *via* a lipid, a liposome or a nanoparticle.

In certain embodiments, the cell that overexpresses LINE-1, which is used in the method of the invention, is an *in vitro* cell. In other embodiments, the cell that overexpresses LINE-1, which is used in the method of the invention, is an *in vivo* cell.

These and other objects, advantages and features of the present invention will become apparent to those of ordinary skill in the art having read the following detailed description of the preferred embodiments.

Brief Description of the Figures

Figure 1. (A) A5 construct map. Forward AluY8a is followed by antisense humanized mono mere clover, antisense humanized HSV-TK interrupted by forward tetrahymena intron, antisense Cytomegalovirus (CMV) mutagenesis has been

introduced to eliminate pol III termination signal found in WT promoter and finely forward poly A (50 bp) ended with polIII termination signal 5xT. **(B) A2 construct map.** Forward AluYa5 is followed by antisense mono mere clover, antisense HSV-TK intrupted by forward tetrahymena intron, antisense Simian vacuolating virus 40 (SV40) promoter and finely forward poly A (50 bp) ended with polIII termination signal 5xT.

Figure 2. LINE-1 ORF1 and ORF2 Relative Expression. Lu1205 and Mel501 are melanoma cells, H3A and H1 are non-melanoma cells, MCF7 and T47D are breast cancer cells, MCF10A are normal breast epithelial cells, C19, A-549 and Calu-1/2 are tongue cancer cells, WI-38 are normal tongue fibroblast, Caco-2, HT29, Colo-320 and SW-320 and SW-620 are colon cancer cells. Total RNA was isolated using RNA purification kit (Macherey-Nagel) according to manufacturer's protocol. 1 μg RNA from each sample was reverse transcribed into cDNA using SuperScript II (invitrogen), qPCR was carried out using SYBR Green PCR kit (Roche) on the LightCycler 480 system (Roche). The signals were standardized on input DNA and normalized with ß-Actin houskeeping gene. Each point represents the average from three independent experiments (n=3).

Figure 3. (A) Schematic representation of an Alu element (in dark gray) flanked with the HSV-TK gene (gray boxes), in which the HSV-TK gene placed in backward orientation with a promoter (Pr) is rendered inactive by the presence of an autocatalytic *Tetrahymena* (Tet) intron (blue), which should be spliced out in the transposition RNA immediate (middle). Expected structure of the resulting *de novo* Alu insertion containing *HSV-TK* is shown (bottom). **(B) HSV-TK expression after Alu_HSV-TK transposition.** Immuno-staining on HEK293 cells. In the upper panel, HEK cells are co-transfected with HSV-TK and GFP expression vectors. In the lower panel HEK cells are transfected with the A2 construct. An *Arrow* indicates intercellular clusters of HSV-TK and GFP and their colocalisation. Bars. 40 μm.

Figure 4. HSV-TK genomic integration. DNA Fluorescence In Situ Hybridization in HEK293 cells transfected with A2 and GFP expression vector, the middle panel corresponds to non-transfected cells. An *Arrow* indicates genomic luci of HSV-TK. Bars. 5 μm.

Figure 5. Efficiency of the concept. Cell death measurement and ORF1p expression in different cell lines. **(A)** FACS analysis of the global cell death measurements (Annexin V and IP). MCF7 breast cancer cells, HT-29 colon cancer, A172 and U87 are glioblastoma cell lines, WI-38 normal immortalized fibroblast. NT: non transfected, GCV: ganciclovir, A2: construct Alu-HSV-TK. **(B)** Positive control of apoptosis induction with U87 and A172 cell lines. **(C)** Western Blot showing the ORF1 protein expression in the different tested cell lines. Actin (lower band in the panel) is used as a loading control.

Figure 6. ORF1 protein expression. Western blot showing ORF1 expression in MCF7 and MDA-MB-231 cell lines, whereas Hela cells do not show any ORF1 protein signal. HEK cell line transfected with L1 expression vector used as control.

Figure 7. (A) Apoptosis induction in HSV-tk (A2) transfected MCF7 cell line. Gancyclovir treatment of transfected MCF7 cell line induced significant apoptosis. NT: non transfect, NT+ GCV: non transfected treated, GFP+GCV: transfected with GFP treated and A2: transfected with HSV-tk non-treated MCF7. **(B) Cell viability**.

Figure 8. Apoptosis induction in HSV-tk (A2) transfected HCT116 cell line. Apoptosis induced by Gancyclovir treatment in HCT116 cell line is less significant compering to MCF7 cell line. NT: non transfect, NT+ GCV: non transfected treated, GFP+GCV: transfected with GFP treated and A2 transfected with HSV-tk non treated HCT116.

Figure 9. ORF1 protein expression screening in several cancer cell lines. Upper panel: western blot showing that ORF1 is expressed in ScaBER cell line (Bladder), Caov3 cancer cell line of ovary, whereas SVG40 normal ovary cell line does not express ORF1. Lower panel: IMR-90 normal lung cell line does not display any signal of ORF1 expression, whereas NCI-H1975, Calu6 and NCI-H1435 lung cancer cell lines express ORF1. A similar expression but less abundant is observed in Hs746m stomach cell line, while no ORF1 expression occurs in pancareatic Panc1 and Panc3 cell lines. Abundant expression was also found in pharynx FaDu and ORL3 cell lines. finally ORF1 is also expressed in bladder TCC-SUP cell line.

Definitions

Throughout the specification, several terms are employed that are defined in the following paragraphs.

As used herein, the term "***gene therapy***" refers to transcription, or transcription and translation, of exogenously supplied nucleic acid to prevent, palliate and/or cure a disease or diseases. The term "***cancer gene therapy***" is used when the disease is a cancer.

By "***gene therapy vector***" is meant a vector useful for gene therapy. Gene therapy vectors carry a gene, or genetic material, of interest that is useful for gene therapy. The gene therapy vectors are able to be transferred to the cells of a mammal, *e.g.*, a human, and are able to express the gene of interest in such cells so as to effect gene therapy.

The terms "***expression cassette***", "***expression construct***" and "***construct***" are used herein interchangeably. They refer to a nucleotide sequence capable of directing the transcription, or transcription and translation, of a heterologous coding sequence (*i.e.*, a gene or nucleic acid sequence of interest) and the heterologous coding sequence to be expressed. An expression cassette comprises regulatory elements operably linked to a heterologous coding sequence so as to achieve expression of the protein product encoded by said heterologous coding sequence in the cell. Thus, an expression cassette comprises a complete set of control sequences including initiation, promoter and termination sequences which function in a cell when they flank a coding sequence in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired coding sequence. Preferably, a cassette has its 3' and 5' ends adapted for ready insertion into a vector, *e.g.*, it has retriction endonucleases sites at each end.

The term "***vector***", as used herein, refers to a nucleic acid molecule capable of transporting another nucleic acid molecule (*e.g.*, an expression cassette) to which it has been linked. The term "***expression vector***" includes any vector (*e.g.*, a plasmid, cosmid or phage chromosome) containing a gene construct in a form suitable for expression in a cell. The terms "***plasmid***" and "***vector***" are often used interchangeably, as a plasmid is a commonly used form of vector. However, the invention is intended to include other vectors which serve equivalent functions, such as viral and non-viral vectors, including

adenovirus vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, and the like.

The term "***heterologous***", as used herein to characterize a DNA sequence (*e.g.*, a coding sequence), refers to a nucleotide sequence which is not endogenous to the cell into which it is introduced. Heterologous DNA includes a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA also includes a nucleotide sequence which is naturally found in the cell into which it is introduced and which contains some modification relative to the naturally occurring sequence.

As used herein, the term "***gene***" refers to a polynucleotide (*e.g.*, DNA) that encodes a discrete macromolecular product, be it a RNA or a polypeptide, and may include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. As more than one polynucleotide may encode a discrete product, the term also includes alleles and polymorphisms of a gene that encode the same product, or a functionally associated (including gain, loss, or modulation of function) analog thereof. The term "gene" encompasses both cDNA and genomic forms of a given gene. The term "***gene expression***" refers to the process by which RNA and proteins are made from the instructions encoded in genes. Gene expression includes transcription, or transcription and translation, of nucleic acid material.

By "***gene of interest***" is meant any coding sequence that is to be transcribed, or transcribed and translated, within a host organism. In the context of the present invention, genes of interest include not only suicide genes but also any "***therapeutic gene***", *i.e.*, any gene that encodes a pharmaceutically active protein useful for the treatment of cancer. Genes of interest also include antisense DNA molecules or other DNA elements intended to be transcribed but not translated.

The term "***reporter gene***" indicates a gene sequence that encodes a reporter molecule (*e.g.*, an enzyme). A "reporter gene" or "reporter molecule" may be detectable in any of a variety of detection systems including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. Specific examples include *E. coli* β-galactosidase gene

(available from Pharmacia Biotech, Pistacataway, NJ), green fluorescent protein (GFP) (commercially available from Clontech, Palo Alto, CA), the human placental alkaline phosphatase gene, and the chloramphenicol acetyltransferase (CAT) gene. Other reporter genes are known to the art and may be employed.

The terms "***regulatory element***" and "***regulatory sequence***" are used herein interchangeably. They refer to a nucleotide sequence which controls some aspect of the expression of nucleic acid sequences (*i.e.*, the RNA transcription, the processing, the stability and/or the subsequent translation of the transcribed RNA). Regulatory elements include, but are not limited to, promoter, enhancers, and other expression control elements such as polyadenylation sequences, 5'- and 3'-untranslated regions (UTRs), intron sequences, splicing signals, termination signals, silencing regions, post transcriptional regulatory elements, and the like.

The term "***promoter***", as used herein, refers to a nucleotide sequence at which the intiation and rate of transcription of a coding sequence is controlled. By "promoter" is meant a minimal sequence sufficient to direct transcription of an operably-linked gene of interest, antisense sequence, etc... The promoter contains the site at which RNA polymerase binds and also contains sites for the binding of regulatory factors (such as repressors and transcription factors). The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "*enhancers*" As used herein, the term "***enhancer***" refers to a polynucleotide that can stimulate promoter activity and thereby increase the level of expression of an operably linked gene of interest, antisense sequence, etc... relative to the level of expression that occurs in the absence of the enhancer. Enhancers may increase expression of operably linked sequences in a tissue specific or temporal manner. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses. Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal. The term "***poly A site***" or "***poly A sequence***, as used herein, denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the

recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be heterologous or endogenous. An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. As used herein, the term "***untranslated region (UTR)***" refers to a sequence located at the 3'- and 5'-ends of the coding sequence, and that contains a polyadenylation signal as a transcription termination region, and the like. The term "***intron***" refers to an untranslated nucleotide sequence located between exons translated to a protein after transcription. The transcribed mRNA precursor is converted into a mature mRNA after the introns are removed through splicing. The term "intron", as used herein, is also intended to comprehend a splicing donor, a splicing acceptor, a triple guanine repeat sequence (G-triple motif) and/or a branch sequence.

The terms "***operably linked***", "***in operable order***" and "***in operable combination***" are used herein interchangeably and refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing transcription of a given gene and/or the synthesis of a desired protein molecule is produced. Thus, two nucleotide sequences, one being a regulatory sequence, are operably linked if they are associated in a manner that allows the regulatory sequence to affect expression of the other nucleotide sequence. For example, a promoter (or enhancer) is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the nucleotide sequences being linked are typically contiguous. However, as enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not directly flanked and may even function in trans from a different allele or chromosome.

As used herein, the term "***subject***" refers to a mammal that can suffer from cancer, but may or may not have the disease. In many embodiments of the present invention, the subject is a human being. In such embodiments, the subject is often referred to as an "***individual***" or a "***patient***". The terms "subject", "individual" and "patient" do not denote a particular age, and thus encompass newborns, children, teenagers, and adults.

The term "***treatment***" is used herein to characterize a method or process that is aimed at (1) delaying or preventing the onset of a disease or condition (here a cancer); (2) slowing down or stopping the progression, aggravation, or deterioration of the symptoms of the disease or condition; (3) bringing about amelioration of the symptoms of the disease or condition; or (4) curing the disease or condition.

A "***pharmaceutical composition***" is defined herein as comprising a therapeutically effective amount of at least one therapeutic agent (*e.g.*, an Alu-retrotansposon-based vector according to the invention), and at least one pharmaceutically acceptable carrier or excipient.

The term "***pharmaceutically acceptable carrier or excipient***" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredient(s) and which is not excessively toxic to the host at the concentration at which it is administered. The term includes solvents, dispersion, media, coatings, antibacterial and antifungal agents, isotonic agents, and adsorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art (see for example "*Remington's Pharmaceutical Sciences*", E.W. Martin, 18th Ed., 1990, Mack Publishing Co.: Easton, PA, which is incorporated herein by reference in its entirety).

As used herein, the term "***therapeutically effective amount***" refers to any amount of a therapeutic agent or composition thereof that is sufficient to fulfil its intended purpose(s), *e.g.*, a desired biological or medicinal response in a cell, tissue, system or subject. For example, in certain embodiments of the present invention, the purpose(s) may be to treat a cancer.

Detailed Description of the Invention

The present invention provides novel recombinant Alu-retrotransposon-based expression cassettes and vectors and their use for specific and stable incorporation of a gene (or nucleic acid molecule) of interest into cancer cells for cancer gene therapy. The invention exploits the selective expression of LINE-1 elements in tumor cells to provide selectivity and hence safety of treatment.

I - Alu-Retrotransposon-Based Constructs and Vectors

An expression cassette according to the invention comprises an Alu retrotransposon flanked with a gene (or genetic material or nucleic acid sequence) of interest, wherein the gene (or genetic material or nucleic acid sequence) of interest is to be delivered to a cancer cell in a subject, and more specifically to a cancer cell which expresses LINE-1.

A. Alu-Retrotransposons

The terms "***Alu retrotransposon***", "***Alu element***", "***Alu retroelement***", "***SINE/Alu retrotransposon***", "***SINE/Alu element***", "***SINE/Alu retroelement***" are used herein interchangeably. As already indicated above, Alu retrotransposons are the most abundant interspersed repeats in the human genome. They are a family of short interspersed nuclear elements (SINEs) that use the reverse transcriptase and nuclease encoded by long interspersed nuclear elements (LINEs) to integrate into the host genome (Dewannieux *et al.*, Nature Genetics, 2003, 35: 41-48). Alu elements are found in the human genome in a number of more than a million copies, covering about 10% of its total length. Phylogenetic studies showed that Alu elements derived from the 7SL RNA gene (Ullu and Tschudi, Nature, 1984, 312(5990): 171-172).

Alu typical sequences are about 300 nucleotides long and are classified into subfamilies according to their relative ages. They have a dimeric structure and are composed of two similar but distinct monomers: left and right arms of about 100 and about 200 nucleotides long, respectively, held together by an A-rich linker and terminated by a short poly(A) tail. Each of the Alu subunits originated from 5' and 3' terminal segments of 7SL RNA. Alu sequences contain internal Pol III promoter elements and they are CG and CpG rich.

Alu sequences within the human genome can be divided into subfamilies based upon diagnostic mutations shared by subfamily members, and they appear to be of different genetic ages. Indeed, the majority of the Alu elements were inserted into the primate genome 35 to 65 million years ago, but certain subfamilies of Alu elements are relatively very new and suspected to be still evolving (Bennett *et al.*, Genome Research, 2008, 18: 1875-1883). The earliest Alu elements were the J subfamily, followed by S subfamilies (that include Sx, Sq, Sp and Sc), and followed by the more recent Y subfamilies. AluY subfamilies include, but are not limited to, AluYc1, AluYc2,

AluYa5, AluYa5a2, AluYa8, AluYd8, AluYb8 and AluYb9, which are considered to be very active due, with Ya5 and Yb8 being the most dominant in humans.

Thus, in certain preferred embodiments, the Alu retrotransposon present in a construct according to the present invention belongs to the AluY subfamily, and preferably to the AluYa5 or to the AluYa8 subfamilies.

In certain embodiments, the Alu retrotransposon present in the construct according to the present invention belongs to the AluYa8 subfamily of retrotransposon and has the sequence set forth in SEQ ID NO: 1, wherein SEQ ID NO: 1 is:
GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAG
GCGGGCGGATCACGAGGTCAGGAGATCGAGACCATCCCGGCTAAAACGGT
GAAACCCCGTCTCTACTAAAACTACAAAAAATAGCCGGGCGTAGTGGCGGG
CGCCTGTAGTCCTAGCTACTTGGGAGGCTGAGGCAGGAGAATGGCGTGAAC
CCGGGAGGCGGAGCTTGCAGTGAGCCGAGATCCCGCCACTGCACTCCAGCC
TGGGCGACAGAGCGAGACTCCGTCTCA.

In other embodiments, the Alu retrotransposon present in the construct according to the present invention is belongs to the AluYa5 subfamily of retrotransposons and has the sequence set forth in SEQ ID NO: 2, wherein SEQ ID NO: 2 is:
GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAG
GCGGGCGGATCACGAGGTCAAGAGATCGAGACCATCCCGGCTAAAACGGT
GAAACCCCGTCTCTACTAAAAATACAAAAAAATTAGCCGGGCGTGGTAGCG
GGCGCCTGTAGTCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATGGCGTGA
ACCCGGGAGGCGCAGCTTGCAGTGAGCCGAGATCCCGCCACTGCACTCCAG
CCTGGGCGACAGAGCGAGACTCCGTCTCA.

In yet other embodiments, the Alu retrotransposon present in the construct according to the invention has a nucleotide sequence that is at least 80% homologous, preferably at least 85% homologous, and even more preferably at least 90% homologous to SEQ ID NO: 1 or to SEQ ID NO: 2. As used herein, the expression "nucleotide sequence homologous to the sequence SEQ ID NO: 1 or SEQ ID NO: 2" refers to any nucleotide sequence which differs from the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 by substitution, deletion and/or insertion of one nucleotide or of a limited number of nucleotides, at positions such that these homologous nucleotide sequences are active Alu retrotransposons of the AluYa8 subfamily or of the AluYa5

subfamily, respectively. Preferably, a nucleotide sequence homologous to the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 has a percentage identity of at least 90%, preferably of at least 95% (for example 96%, 97%, 98% or 99%), with the sequence SEQ ID NO: 1 or SEQ ID NO: 2, respectively.

The term "percentage identity" or "homology" between two nucleotide sequences is intended to denote a percentage of nucleotides which are identical between the two sequences to be compared, obtained after optimal alignment. This percentage is purely statistical and the differences between the two sequences are distributed randomly and over the entire length of the sequence. The terms "optimal alignment" and "best alignment", which are used interchangeably here, denote the alignment for which the percentage identity determined as described below is the highest. The optimal alignment of the sequences, required for the comparison, can be produced manually or by means of computer programs (GAP, BESTFIT, BLASTP, BLASTN, FASTA and TFASTA, which are available, for example, either on the NCBI website or in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI). The percentage identity between two nucleotide sequences is calculated by determining the number of identical positions for which the nucleotide is identical between the two sequences, by dividing this number of identical positions by the total number of positions compared, and by multiplying the result obtained by 100.

Preferably, in the context of the present invention, a nucleotide sequence homologous to SEQ ID NO: 1 or to SEQ ID NO: 2 hybridizes specifically to the sequence complementary to the sequence set forth in SEQ ID NO: 1 or in SEQ ID NO: 2, respectively under stringent hybridization conditions or moderately stringent hybridization conditions. Said hybridization conditions can be established by means of conventional protocols described, for example, in Sambrook *et al.*, "Molecular Cloning – A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1989, or Ausubel *et al.*, "Current Protocols in Molecular Biology", Green Publishing Associates and Wiley Interscience, 1989.

B. Gene (or Genetic Material) of Interest

As indicated above, the Alu-retrotransposon system according to the present invention is particularly well suited for the delivery of suicide genes in cancer gene therapy. Thus, in certain preferred embodiments of the present invention, the gene of

interest is a suicide gene. However, the present invention also contemplates the use of other genes, in particular genes that encode a pharmaceutically active protein useful in the treatment of cancer.

In the construct, the gene of interest is placed in the opposite transcriptional orientation to the Alu element (*i.e.*, positioned in the antisense orientation with respect to the direction of the Alu transcription). Furthermore, in the construct, the gene of interest is interrupted by a small autocatalytic intron, which is placed in the opposite transcriptional orientation to the gene of interest but in the same orientation as the Alu element. As used herein, the term "***autocatalytic intron***" has its art understood meaning and refers to an intron that catalyze its own excision from mRNA, tRNA or rRNA precursors. The small autocatalytic intron may be the autocatalytic Tetrahymena (Tet) intron or any other intron belonging to group I introns. The intron will be spliced out in the intermediate transposition RNA, allowing expression of the gene of interest only after integration in the genome.

Suicide Genes. In certain embodiments, the gene of interest is a suicide gene. A suicide gene may be any gene that expresses a product that is lethal to the cell expressing the suicide gene. Most suicide genes code for viral or bacterial enzymes that convert an inactive drug into toxic antimetabolites that inhibit the synthesis of nucleic acid leading to cell death. Suicide genes must be introduced into the cells in ways that ensure their uptake and expression by as many cancer cells as possible, while limitating their expression by normal cells. Thus, the Alu-retrotransposon-based vectors of the present invention are particularly well suited for the delivery of suicide genes.

Suicide genes are well known in the art and include, without limitation, the thymidine kinase gene (HSV-TK) of herpes simplex virus, cytosine deaminase (CD) gene of *Escherichi coli*, and the human carboxylesterase gene (CE) isoform1. HSV-TK converts non-toxic Ganciclovir (GCV) into its toxic active form. HSV-TK has a high affinity for GCV, which allows it to catalyze the phosphorylation of GCV into its monophosphate form, which is further converted into its di- and triphosphate derivatives by cellular kinases. DNA polymerase then incorporates GCV-triphospahte into replicating DNA, leading to cell death by polymerase inhibition and induction of apoptosis. CE converts the prodrug 5-fluorocytosine (5-FC) into a toxic agent, 5-fluorouracil (5-FU), which inhibitrs RNA and DNA synthesis. CE converts the prodrug

CPT-11 into its active form, SN-38, which is a potent mammalian topoisomerase I inhibitor.

Other examples of suicide gene systems include the hepatic cytochrome P450-2B1 gene, the nitroreductase gene, the varicella-zoster virus thymidine kinase (VZV-TK) gene, the purine nucleoside phosphorylase (PNP) gene, the beta-galatosidase gene, the linamarase gene, the horseradish peroxidase gene, the carboxypeptidase A gene and the carboxypeptidase G2 gene.

Other Therapeutic Genes. The present invention contemplates the use of a variety of different therapeutic transgenes other than suicide genes. Examples of therapeutic genes suitable for use in the practice of the present invention include, but are not limited to, genes that code for a tumor suppressor, an inhibitor of apoptosis, a toxin, or a cytokine and cell cycle regulatory genes.

Tumor Suppressor Genes. Tumor suppressor genes function to inhibit excessive cellular proliferation. The inactivation of these genes destroys their inhibitory activity, resulting in unregulated proliferation. Many cancers have been shown to have intactivated tumor suppressor genes. Tumor suppressor gene therapy is that part of gene therapy which aims to restor the function of a tumor suppressor gene lost or functionally inactivated in cancer cells. Reintroduction of known wild-type tumor suppressors into tumor cells *in vitro* has been shown to cause an acute change in cell physiologiy and gene expression, resulting in growth arrest or cell death. Tumor suppressor genes which have the capacity to efficiently induce tumor cell death are good candidates for tumor suppressor gene therapy. Examples of such tumor suppressor genes are p53 (tumor protein p53, also known as cellular tumor antigen p53, phosphoprotein p53 or tumor suppressor p53), p16 (cyclin-dependent kinase inhibitor 2A also known as multiple tumor suppressor 1) and C-CAM. Other tumor suppressor genes include, but are not limited to, retinoblastoma (Rb), PTEN (phosphate, tensin homologue), pVHL (von Hippel-Lindau tumor suppressor), APC (Adenomatous polyposis coli (APC) also known as deleted in polyposis 2.5 (DP2.5)), CD95 (cluster of differentiation 95 also known as the FAS receptor (FasR), apoptosis antigen 1 (APO-1 or APT), or tumor necrosis factor receptor superfamily member 6), ST5 (Suppression of tumorigenicity 5), YPEL3 (Yippee like three), ST7 (Suppressor of tumorigenicity

protein 7) and ST14 (Suppressor of tumorigenicity 14 protein, also known as matriptase).

Apoptosis Inhibitor Encoding Genes. Apoptosis, or programmed cell death, is an essential process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis. Thus, the therapeutic gene of interest may be a gene encoding a proapoptotic protein such as p53 (tumor protein p53, also known as cellular tumor antigen p53, phosphoprotein p53 or tumor suppressor p53), BAX (Bcl-2–associated X protein), Bak (Bcl-2 homologous antagonist/killer), Bik (Bcl-2-interacting killer), Bim (Bcl-2-like protein 11 or BCL2L11), Bid (BH3 interacting-domain death agonist), Bad (Bcl-2-associated death promoter), HRK (Activator of apoptosis harakiri), Apo2L (Apo2 ligand also known as TRAIL), and the like.

Toxin Encoding Genes. In another embodiment, the therapeutic gene may be a gene encoding a toxin that inhibits tumor cell growth. For example, a toxin encoding gene suitable for use in the present invention may encode gelonin, ricin A Chain, Pseudomonas exotoxin, diphtheria toxin, mitogillin, saporin, ribosome inhibitory protein, and the like.

Cytokine Encoding Genes. In another embodiment, the therapeutic gene may be a gene encoding a cytokine. Cytokines include chemokines, interferons, interleukins, lymphokins, tumor necrosis factors, and colony stimulating factors. Cytokines are proteins made and releasesd by a broad range of host cells in response to the presence of pathogens such as viruses, bacteria, parasites or tumor cells. Cytokines play important roles in inflammatory and immune responses. Some have important effects on tumor growth by augmenting anti-tumor immune response, inducing apoptosis in cancer cells or impairing the formation of new tumoral vessels. Several cytokines have been shown to exert a relevant anti-tumor activity both in experimental models and in humans.

Examples of cytokine encoding genes suitable for use as therapeutic genes of interest in the present invention include, but are not limited to, genes encoding interleukin 2 (IL-2), IL-7, IL-12, IL-15, IL-18, interferon alpha, interferon gamma, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma-induced protein 10 (IP-10), and fms-like tyrosine kinase-*3* (Flt-3) ligand.

Cell Cycle Regulotory Genes. In another embodiment, the therapeutic gene may be a cell cycle regulatory gene, the expression of which in a cancer cells inhibits

proliferation of the cancer cells. For example, a cell cycle regulatory gene suitable for use in the present invention may encode Rb, p53, cell cycle dependent kinase, CDK kinase, cyclin or a constitutively active Rb gene product, or an antisense RNA.

Nucleic Acids of Interest. The present invention also provides a new and effective method for delivering RNA interfering (RNAi) agents using Alu retrotransposon-based vectors for cancer therapy. As used herein, the term "***RNA interference***" (or "***RNAi***") has its art understood meaning and refers to a biological process in which RNA molecules silence, inhibit or down regulate gene expression by causing the destruction/degradation/cleavage of specific mRNA molecules or by blocking the translation thereof. RNAi technology can be directed against numerous aberrant genes, including those that allow proliferation of tumor cells. A variety of strategies can be used to inhibit cancer. These include the inhibition of overexpressed oncogenes, blocking cell division by interfering with cyclin E and related genes or promoting apoptosis by suppressing antiapoptotic genes. RNAi against multidrug resistance genes or chemoresistance targets may also provide useful cancer treatments. See for example, the following reviews on RNA interference for the treatment of cancer by gene therapy: Izquierdo, Cancer Gene Ther., 2005, 12: 217-227; Putral *et al.*, Drugs News Perspect., 2006, 19: 317-324; Gartel and Kandel, Biomol. Engl., 2006, 23: 17-34.

An RNAi agent may be any single-stranded RNA (*e.g.*, mature miRNA, ssRNAi oligonucleotides, ssDNAi oligonucleotides) or double-stranded RNA (*i.e.*, duplex RNA such as siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.*, by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the RNAi agent is in the same cell as the target gene or sequence. The term "RNAi agent" thus refers to the single-stranded RNA that is complementary to a target mRNA sequence (or circRNA sequence) or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. A RNAi agent may have substantial or complete identity to the target gene mRNA (or circRNA) sequence, or may comprise a region of mismatch (*i.e.*, a mismatch motif). As used herein, the term "***mismatch motif***" refers to a portion of an RNAi agent sequence that does not have 100% complementarity to its target sequence. An RNAi agent may have at least one, two, three, four, five, six, or more mismatch

regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides. Consequently, the term "RNAi agent" refers to a RNA molecule comprising a strand having a sequence sufficiently complementary to a target mRNA (or circRNA) sequence to direct target-specific RNA interference (RNAi) thereby inhibiting or down-regulating the expression of the target gene. An RNAi agent can comprise naturally occurring RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end of the molecule or to one or more internal nucleotides of the RNAi, including modifications that make the RNAi agent resistant to nuclease digestion.

In certain preferred embodiments of the present invention, an RNAi agent is a siRNA (small interfering RNA), a shRNA (short hairpin RNA), a micro-RNA (micro RNA), or an aiRNA (asymmetric interfering RNA). The development of any type of RNAi agent capable of specifically silencing, inhibiting or down-regulating the expression of a given target gene is within the capabilities of one skilled in the art. Thus, for example, siRNAs are usually designed against a region 50-100 nucleotides downstream the translation initiator codon, whereas 5'UTR (untranslated region) and 3'UTR are usually avoided. The chosen siRNA target sequence should be subjected to a BLAST search against EST database to ensure that only the desired gene is targeted. Various products are commercially available to aid in the preparation and use of siRNA.

One skilled in the art will recognize that other genes and nucleic acid molecules than those described herein can efficiently be delivered by an Alu-retrotransposon system of the present invention.

C. Regulatory Elements

An Alu-retrotransposon-based cassette according to the invention comprises any regulatory element necessary for the transcription, or the transcription and translation, of the gene (or genetic material) of interest in the host cell. In particular, regulatory elements include tissue specific promoters, different viral promoters, insulators,

enhancers, initiation signals, splicing signals, termination signals, polyadenylation signals.

Promoters. A promoter is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription of a nucleic acid sequence. A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known examples of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminated deoxynucleotidy transferase gene and the promoter for the SV40 late gene, a discrete element overlying the start site itself helps to fix the place of initiation.

The promoter operably-linked to the gene of interest may be a tissue specific promoter or a constitutive promoter. The term "***tissue specific promoter***" refers to a promoter that is able to express, in a selective manner, nucleic acid sequences to which it is operably linked, in specific tissues of the host organism. The term "***constitutive promoter***" refers to a promoter that is able to expres nucleic acid sequences operably linked to the promoter, in every, or almost every, tissue of the host organism and during the entire development of this organism. Constitutive promoters that can be used in the practice of the present invention include, but are not limited to, immediate early cytomegalovirus (CMV) promoter, herpes simplex virus 1 (HSV1) immediate early promoter, SV40 promoter, lysozyme promoter, early and late CMV promoters, early and late HSV promoters, β-actin promoter, tubulin promoter, Rous-Sarcoma virus (RSV) promoter, heat-shock protein (HSP) promoter and chicken beta-actin promoter with CMV enhancer (CAG) promoter.

Enhancers. In certain preferred embodiment, a construct according to the present invention comprises an enhancer of the Alu element. Preferably, such an enhancer is operably linked to the Alu retrotransposon or any inhancer for polIII promoter. For example, the enhancer may be a 5' enhancer of the Alu-like 7SL RNA gene. In certain embodiments, the 5' enhancer of the Alu-like 7SL RNA gene has the sequence set forth in SEQ ID NO: 3, wherein SEQ ID NO: 3 is:

GATCCCCCCCCCCCCGCTCCCCAATGACGTAACTGCCCTGCAGCTTCTAGTAG CTTTTCGCAGCGTCTCCGACC.

Initiation Signals. A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG intiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in frame" with the reading frame of the desired coding signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription elements.

Splicing Sites. Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression.

Termination Signals. An Alu-retrotransposon-based construct of the present invention will generally comprise at least one termination signal. A termination signal or terminator is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments, a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to be more stable and are translated more efficiently. Thus, in certain embodiments involving eukaryotes, it is preferred that the terminator signal comprise a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any terminator of transcription known in the art, including but not limited to, the viral termination

sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence.

Polyadenylation Signals. In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. For example, the polyadenylation signal may be the SV40 polyadenylation signal, which is known to function in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

D. Gene Delivery Techniques

An Alu-retrotransposon based construct of the present invention may be delivered using any gene delivery system used in cancer gene therapy. Vector constructs for delivering nucleic acid molecules can be viral, non-viral or physical (see, for example, Rosenberg *et al.*, Science, 1988, 242: 1575-1578, and Wolff *et al.*, Proc. Natl. Acad. Sci. USA, 1989, 86: 9011-9014). Examples of gene delivery systems include, but are not limited to, liposomal delivery vectors, viral delivery vectors, drug delivery vectors, chemical carriers, polymeric carriers, lipoplexes, polyplexes, dendrimers, microbubbles (ultrasound contrast agents), nanoparticles, emulsion, or other appropriate transfer vectors. Discussions of methods and compositions for use in gene therapy are provided in Eck *et al.*, in Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, Hardman *et al.* eds., McGray-Hill, New York, 1996, Chapter 5, pp. 77-101; Wilson, Clin. Exp. Immunol., 1997, 107 (Suppl. 1): 31-32; Wivel *et al.*, Hematology/Oncology Clinics of North America, Gene Therapy, S. L. Eck, ed., 1998, 12(3): 483-501; Romano *et al.*, Stem Cells, 2000, 18: 19-39.

Viral Vectors. In certain embodiments, the Alu-retrotransposon construct of the present invention is inserted into a viral vector. Viral vectors include adenoviruses, adeno-associated viruses (AAVs), retroviruses and lentiviruses. Both human and non-human viral vectors can be used and the recombinant viral vectors can be replication-defective in humans. Preferred viral vectors are the adenoviruses and adeno-associated viruses, which are single-stranded DNA viruses that have already been approved fro human use in gene therapy. Options for gene delivery viral constructs are well known (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley

& Sons, New York, 1989; Kay *et al.*, Nat. Medic., 2001, 7: 33-40; and Walther and Stein, Drugs, 2000, 60: 249-271).

Adenovirus Vectors. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanded with the high efficieny of gene transfer afforded by these vectors. The term "*adenovirus expression vector*" is meant to include those constructs that contain adenovirus sequences sufficient to (a) support packaging of the contruct and (b) to ultimately express a construct that has been cloned therein. Of course, in the context of an antisense construct, expression does not require that the gene product be synthesized.

Adenovirus is a non-enveloped, nuclear DNA virus with a genome of about 36 kb, which has been well-characterized (Hurwitz, Adenoviruses Virology, 3rd edition, Fields *et al.*, eds., Raven Press, New York, 1996; Hitt *et al.*, Adenovirus Vectors, The Development of Human Gene Therapy, Friedman, *ed.*, Cold Spring Harbor Laboratory Press, New York, 1999).

Recombinant adenoviral vectors have several advantages for use as gene delivery vehicles, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, structural stability, and the potential to carry large inserts. Adenoviral vectors with deletions of various adenoviral gene sequences, such as pseudoadenoviral vectors (PAVs) and partially-deleted adenoviral (termed "DeAd"), have been designed to take advantage of the desirable features of adenovirus which render it a suitable vehicle for delivery of nucleic acids to recipient cells.

Adeno-Associated Vectors (AAVs). AAV is a single-stranded human DNA parvirus whose genome has a size of about 4.6 kb. It was discovered as a contaminiation of adenoviral stocks. AAV is an attractive vector system for use in the present invention as it has a high frequency of integration. There are other advantages to the use of AAV for gene transfer. The host range of AAV is broad. Moreover, unlike retroviruses, AAB can infect both quiescent and dividing cells. In addition, AAV has not been associated with human disease, obviating many of the concerns that have been raised with retrovirus-derived gene transfer vectors. Details concerning the generation and use of recombinant AAV vectors are described in U.S. Patent Numbers 5,139,942 and 4,797,368 (each of which is incorporated herein by reference).

Retroviral Vectors. Retroviruses vectors are a common tool for gene delivery (Miller, Nature, 1992, 357: 455-460). The ability of retrovirus vectors to deliver an unrearranged, single copy gene into a broad range of rodent, primate and human somatic cells makes retroviral vectors well suited for transferring genes to a cell. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription. The resulting DNA is then stably integrated into cellular chromosomes as a provirus directs synthesis of viral proteins. The integration results in the retention of the viral gene sequence in the recipient cell and its descendants. The retroviral genome contains three genes, *gag*, *pol* and *env* that encode capsid proteins, polymerase enzyme and envelop components, respectively. A sequence found upstream from the *gag* gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These sequences contain strong promoter and enhancer sequences and are also required for integration into the host cell genome.

Retroviruses have been approved for human gene therapy trials. Retroviral vectors are able to infect a broad variety of cell types. Most useful are those retroviruses that are replication-deficient (*i.e.*, capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression viral constructs have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are known in the art.

Lentiviruses. Lentiviruses are complex retroviruses which, in addition to the common retroviral genes, *gag*, *pol*, and *env*, contain other genes with regulatory or structural function. The higher complexity enables the lentivirus to modulate the life cycle thereof, as in the course of latent infection. Lentiviral vectors are well known in the art and their use for gene therapy has been described (see, for example, U.S. Pat. Nos. 6,013,516 and 5,994,136). Examples of lentiviruses include, but are not limited to,

the Human Immunodeficiency Virus (HIV-1, HIV-2) and the Simian Immunodeficiency Virus (SIV). Lentiviral vectors have been generated by multiply attenuating the the HIV virulence genes, for example, the genes *eng*, *vif*, *vpr*, *vpu* and *nef* are deleted making the vector biologically safe.

Other Viral Vectors. Other viral vectors that can be used in accordance with the present invention. Vectors derived from viruses such as vaccina virus, sindbis virus, cytomegalovirus (CMV) and herpes simplex virus (HSV) may be employed. They offer several attractive features for infection of various mammalian tumor cells. HSV vectors deleted of one or more immediate early genes (IE) are advantageous because they are generally non-cytotoxic, persist in a state similar to latency in the target cell, and afford efficient target cell transduction.

Non-Viral Vectors. In addition to viral vector-based methods, non-viral methods may also be used to deliver an Alu-retrotransposon construct according to the invention into a cancer cell. A review of non-viral methods of gene delivery is provided, for example, in Nishikawa and Huang, Human Gene Ther., 2001, 12: 861-870; Morille et al., Biomaterials, 2008, 29: 3477-3496; Elsabahy *et al.*, Curr. Drug Deliv., 2011, 8: 235-244; Wang *et al.*, Curr. Pharm. Biotechnol., 2013, 14: 46-60; and Yin *et al.*, Nature Rev. Genet., 2014, 15: 541-555.

Non-viral delivery systems include plasmid constructs, which are well known in the art. Other non-viral delivery systems include, but are not limited to, liposomes, biocompatible polymer carriers, lipoplexes, polyplexes, lipopolysaccharides, dendrimers, microbubbles, metal particles, and nanoparticles. These delivery systems are well known in the art.

Gene transfer systems that combine viral and non-viral components may also be used in the practice of the present invention. Such hybrid systems have been reported (Cristiano *et al.*, Proc. Natl. Acad. Sci. USA, 1993, 90: 11548; Wu *et al.*, J. Biol. Chem., 1994, 269: 11542; Wagner *et al.* Proc. Natl. Acad. Sci. USA, 1992, 89: 6099; Yoshimura *et al.*, J. Biol. Chem., 1993, 268: 2300; Curiel *et al.*, Proc. Natl. Acad. Sci. USA, 1991, 88: 8850; and Kupfer *et al.*, Human Gene Ther., 1994, 5: 1437). The reported combinations generally involve either covalent attachment of an adenovirus to a gene delivery system or co-internalization of unbound adenovirus with cationic lipid DNA complexes. Other methods include virosomes which combine liposomes with an

inactivated HIV or influenza virus, and systems that involve mixing viral vectors with cationic lipids or hybridizing viruses.

E. Preparation of Constructs and Vectors

Irrespective of the components of the construct or vector, one skilled in the art will understand that the construct or vector may be prepared using any of a variety of suitable methods, the method used for preparing the expression construct or vector being a non-critical or limiting element of the invention. The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are well known to the skilled worker and are explained fully in the literature (see, for example, Sambrook, Fritsch and Maniatis, "*Molecular Cloning: A Laboratory Manual*", 1989, Cold Spring Harbor Laboratory: Cold Spring Harbor, Silhavy, Berman, and Enquist, "*Experiments with Gene Fusions*", 1984, Cold Spring Harbor Laboratory: Cold Spring Harbor; F.M. Ausubel *et al.*, "*Current Protocols in Molecular Biology*", 1989, John Wiley & Sons: New York; "*DNA Cloning: A Practical Approach*", Volumes I and II, Glover, ed., 1985; "Oligonucleotide Synthesis", M.J. Gait, ed., 1984; "*Nucleic Acid Hybridization*", Hames & Higgins, eds., 1985; "*Transcription and Translation*", Hames & Higgins, eds., 1984; "*Animal Cell Culture*", Freshney, ed., 1986; "*Immobilized Cells and Enzymes*", IRL Press, 1986; Perbal, "*A Practical Guide to Molecular Cloning*", 1984).

Funtional Tests of the Vector. Vectors containing the construct according to the present invention will routinely be tested after they have been constructed to confirm that the vector is replication-competent and non-transforming. These tests will assure that sequences included in the vector do not interfere with the functioning of the cassette. Replication competence is usually tested by transfecting a population of non-transformed cells of the target cell type with the vector and monitoring episomal DNA production by Southern blot.

F. Specific Alu-Retrotransposon Vectors of the Invention

The present invention provides two Alu-retrotransposon/HSV-TK vectors.

The first Alu-retrotransposon/HSV-TK vector (pUC57 simple A5) has the sequence set forth in SEQ ID NO: 4. It comprises forward AluY8a followed by

antisens humanized mono mere clover, antisense humanized HSV-TK interrupted by forward tetrahymena intron, antisense Cytomegalovirus (CMV) mutagenesis has been introduced to eliminate pol III termination signal found in WT promoter and a forward poly A (50 bp) ended with polIII termination signal 5xT.

The second vector Alu-retrotransposon/HSV-TK vector (pUC57 A2) has the sequence set forth in SEQ ID NO: 5. It comprises forward AluYa5 followed by antisens mono mere clover, antisense HSV-TK intrupted by forward tetrahymena intron, antisense Simian vacuolating virus 40 (SV40) promoter and finely forward poly A (50 bp) ended with polIII termination signal 5xT.

II - Therapeutic Uses and Applications

The present invention relates to novel therapeutic tools for gene therapy, in particular for the treatment of cancer by gene therapy. A gene therapy method according to the invention may be used alone or in combination with other cancer therapies known in the art such as, for example, those described below. The Alu-retrotransposon-based vectors of the invention may be used for *in vivo* gene therapy or *ex vivo* gene therapy. Those skilled in the art are well aware of how to apply gene delivery to *in vivo* and *ex vivo* situations. For *in vivo* gene therapy, the retrotransposon-based vector (or a pharmaceutical composition thereof) is directly administered to the subject. In certain preferred embodiments, an Alu-retrotransposon-based vector of the invention is used for *in vivo* gene therapy. Thus, the present invention provides methods for treating a cancer in a subject, comprising a step of administering to the subject an effective amount of a retrotransposon-based vector (or of a pharmaceutical compositonh thereof) according to the invention.

A. Indications

The Alu-retrotransposon vectors of the present invention may be used in therapeutic methods to any disease that is characterized by an over-expression of LINE-1. In particular, the Alu-retrotransposon vectors of the present invention may be used to treat a cancer, and more specifically to treat a cancer expressing LINE-1. Indeed, without wishing to be bound by theory, the present invention exploits the selective expression of LINE-1 elements in tumor cells to provide selectivity and hence a significant safety range of treatment.

Examples of cancers that may be treated using a method of the invention include, but are not limited to, bone cancer, bladder cancer, brain cancer, breast cancer, cancer of the urinary tract, cervical cancer, colon cancer, endometrium cancer, esophageal cancer, eye cancer, gastric cancer, head and neck cancer, hepatocellular cancer, liver cancer, lung cancer, lymphoma and leukemia, mouth cancer, ovarian cancer, pancreatic cancer, pituitary cancer, prostate cancer, rectal cancer, renal cancer, stomach cancer, testicular cancer, thyroid cancer, throat cancer, and uterine cancer.

In certain embodiments, a method of treatment according to the invention is carried out after the cancer to be treated has been identified as a cancer that expresses LINE-1 (*i.e.*, has been tested to determine that the cancer expresses LINE-1). Methods to detect and/or measure levels of LINE-1 expression in biological tissues (including cancer tissues) are known in the art. For example, a northern blot analysis of RNA extracts from the tumor tissue and from corresponding healthy tissues may be performed with a strand-specific RNA probe complementary to the first hundred base pairs of the LINE-1 sequence, as described in Belancia *et al.*, Nucl. Acids Res., 2010, 38: 3909-3922. Another method to detect and quantify LINE-1 levels in human tissues is described in the Examples section below and includes western blot analysis of protein extracts from the tumor tissue and from corresponding healthy tissues. The test is based on the use of primary antibodies that recognize ORF1 and ORF2 proteins. Immuno-staining of tumor sections using antiORF1/2 antibodies can also be used to detect and localise ORF1/2 protein within tumor cells.

A cancer to be treated is identified as a "cancer that expresses LINE-1" if the level of LINE-1 expression in the cancer tissue is higher than the level of LINE-1 expression in the corresponding healthy tissue, *e.g.*, at least 1.2 times higher, or at least 1.5 times higher, or at least 2 times higher, or at least 3, 4, 5, 6, 7, 8, 9, 10 times higher or more.

B. Administration

The methods of treatment of the present invention may be accomplished by administering to the cancer patient a therapeutically effective amount of an Alu-retrotransposon vector, as described herein, or a pharmaceutical composition thereof. The administration may be systemic or local. For practically any tumor, systemic delivery is contemplated. This will prove especially important for attacking

microscopic or metastatic cancer. Where discrete tumor mass, or solid tumor, may be identified, a variety of direct, local and regional approaches may be taken.

Administration may be performed using any of the methods known to one skilled in the art. In particular, administration may be performed using any suitable route that allows introduction of the Alu-retrotransposon vector into a host cell in need of gene therapy. Thus, administration may be performed intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostaticaly, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, rectally, topically, intratumorally, intramuscularly, intraperitoneally, subcutaneously, intravesicularlly, mucosally, intrapericardially, orally, topically, locally, using aerosol, injection, infusion, continuous infusion, localized perfusion bathing target cells directly, *via* a catheter, *via* a lavage, in cremes, in lipid compositions (*e.g.*, liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference). One skilled in the art knows that these methods of administration may be combined with methods known in the art for facilitating transfection, including without limitation, electroporation, sonoportation, mechanical massage, gene guns, high-velocity bombardment with DNA-coated microprojectiles, conjugation or triparental mating, etc.

In certain embodiments, a therapeutic method according to the invention includes a step of administering an Alu-retrotransposon vector of the invention, or a pharmaceutical composition thereof, to a desired location in need of treatment in a patient receiving gene therapy through administration *via* a selected route. Accordingly, the Alu-retrotransposon vector, or a pharmaceutical composition thereof, may be administered in a variety of ways including, but not limited to, through a vascular system, a duct system, within the lumen of an organ, into an organ, tissue or cell, into a body cavity, into the reproductive system, through the urinary system, intraperitoneally, and through the respiratory system.

For example, the Alu-retrotransposon vector, or a pharmaceutical composition thereof, may be administered into the vascular system. In such embodiments, the Alu-retrotransposon vector, or a pharmaceutical composition thereof, is preferally administered into the cardiovascular system and specifically into one or more chambers

of the heart. Such an administration results in the distribution of the vector to the organs and tissues and cells receiving blood supply from the vessel or the heart. For example, the Alu-retrotransposon vector, or a pharmaceutical composition thereof, can be administered into the left ventricle of the heart, which results in distribution of the vector to the organs supplied by branches of the aorta, for example the celiac, gonodal, superior (cranial) mesenteric and inferior (caudal) mesenteric arteries. Such distribution targets include the liver, ovary, oviduct and testes, among other organs. Administration through the internal mammary artery targets breast cancer cells. Administration into the artery supplying the ovary or to the fallopian tube may be used to supply those tissues. Administration through the portal vein or hepatic artery targets hepatic cells. Intravascular administration further includes administration into any vein, including but not limited to, veins in the systemic circulation and veins in the hepatic portal circulation. Intravascular administration further includes administration into the cerebrovascular system, including the carotid arteries, the vertebral arteries and branches thereof.

Intravascular administration may be coupled with methods known to influence the permeability of vascular barriers such as the blood brain barrier and the blood testes barrier, in order to enhance transfection of cells that are difficult to affect through vascular administration. Such methods are known to those skilled in the art and include use of hyperosmotic agents, mannitol, hypothermia, nitric oxide, alkylglycerols, lipopolysaccharides (Haluska *et al.*, Clin. J. Oncol. Nursing, 2004, 8(3): 263-267; Brown *et al.*, Brain Res., 2004, 1014: 221-227; Weyerbrock *et al.*, J. Neurosurg., 2003, 99(4): 728-737; Erdlenbruch *et al.*, Br. J. Pharmacol., 2003, 139(4): 685-694; Gaillard *et al.*, Microvasc. Res., 2003, 65(1): 24-31; Lee *et al.*, Biol. Reprod., 2004, 70(2): 267-276).

Intravascular administration may also be coupled with methods known to influence vascular diameter, such as use of beta-blockers, nitric oxide generators, prostaglandins and other reagents that increase vascular diameter and blood flow.

Administration to the organ, tissue or site may also occur through non-vascular routes. For example, administration through the urethra and into the bladder targets the transitional epithelium of the bladder. Administration through the vagina and cervix targets the lining of the uterus. Administration to intra-abdominal and intra-thoraric

organs (such as stomach, liver, lung, spleen, kidney) may also occur *via* the organ surface route, for example using an infusion pump.

Depending on the nature and location of the cancer to be treated, one skilled in the art is capable of selecting the most suitable route and method of administration.

C. Dosage

Administartion of an Alu-retrotransposon-based vector of the present invention will be in a dosage such that the amount delivered is effective for the intended purpose. The route of administration, formulation and dosage administered will depend upon the therapeutic effect desired and the severity of the condition to be treated, the presence of any infection, the age, sex, weight and general health condition of the patient as well as upon the potency, bioavailability, and *in vivo* half-life of the Alu-retrotransposon-based vector used, the use (or not) of concomitant therapaies, and other clinical factors. These factors are readily determinable by the attending physician in the course of therapy. Alternatively or additionally, the dosage to be administered can be determined from studies using animal models and/or clinical trials. Adjusting the dose to achive maximal efficacy based on these or other methods are well known in the art and are within the capabilities of trained physicians. As studies are conducted using the inventive Alu-retrotransposon-based vectors, further information will emerge regarding the appropriate dosage levels and duration of treatment.

An Alu-retrotransposon-based vector of the present invention, or a pharmaceutical composition thereof, may be administered in a single administration, multiple administrations, continuously or intermittently. A treatment according to the present invention may consist of a single dose or multiple doses. Thus, administration of an inventive Alu-retrotransposon-based vector, or a pharmaceutical composition thereof, may be constant for a certain period of time or periodic and at specific intervals, *e.g.*, hourly, daily, weekly (or at some other multiple day interval), monthly, yearly (*e.g.*, in a time release form). Alternatively, the delivery may occur at multiple times during a given time period, *e.g.*, two or more times per week; two or more times per month, and the like. The delivery may be continuous delivery for a period of time, *e.g.*, intravenous delivery.

For viral vectors, one generally will prepare a viral vector stock. Depending on the kind of virus and the titer available, one will deliver 1 to 100, 10 to 50, 100 to 1000, or up to $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, or $1x10^{13}$ infectious particles to the patient. Similar figures may be extrapolated for liposomal or other non-viral formulations by comparing relative uptake efficiencies.

In generaly, the amount of Alu-retrotransposon-based vector administered will preferably be in the range of about 1 ng/kg to about 300 mg/kg of body weight per day, for example, between about 100 ng/kg and about 200 mg/kg of body weight per day; or between about 1 μg/kg and about 100 mg/kg of body weight per day, or between about 100 μg/kg and about 50 mg/kg of body weight per day.

D. Combination Cancer Therapy

A wide variety of cancer therapies, known in the art, may be used in combination with a gene therapy method according to the present invention. Thus, in order to increase the effectiveness of the gene therapy method using an expression construct of the invention, it may be desirable to combine this construct with other agents or procedures effective in the treatment of cancer such as, but not limited to, those described below.

For example, one can use a gene therapy method of the present invention with radiation therapy, surgery, chemotherapy, immunotherapy, local heat therapy and/or another gene therapy protocol. Thus, one can use one or several of the standard cancer therapies existing in the art in addition to the gene therapy of the present invention. All therapies other than the gene therapy of the present invention will be referred to as "other cancer therapies".

The other cancer therapy may be performed prior to the gene therapy of the present invention, concurrently with the gene therapy of the present invention, and/or following the gene therapy of the present invention. The other cancer therapy may precede or follow the gene therapy by intervals ranging from minutes to days to weeks. In embodiments where the other cancer therapy and the gene therapy are administered together, one would generally ensure that a significant period of time did not expire between the time of each delivery. In such instances, it is contemplated that one would administer to a patient both modalities within about 12-24 hours of each other and, more

preferably within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6, or 7) to several weeks (1, 2, 3, 4, 5, 6, 7, or 8) lapse between the respective administrations.

It is also conceivable that more than one administration of either of the other cancer therapy and the gene therapy will be required to achieve the desired therapeutic effect (*e.g.*, complete cancer cure). Various combinations may be employed.

Radiotherapy Agents. In certain embodiments, an Alu-retrotransposon vector according to the present invention, or a pharmaceutical composition thereof, is used in combination with radiation therapy. Radiotherapeutic agents and factors include radiation and waves that induce DNA damage, such as, for example, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emission, radioisotopes, and the like. Therapy may be achieved by irradiating the localized tumor site with one of the above described forms of radiations. Any suitable means for delivering radiation to a tissue may be employed in the present invention in addition to external means. For example, radiation may be delivered by first providing radiolabeled antibody that immunoreacts with an antigen of the tumor, followed by delivering an effective amount of the radiolabeled antibody to the tumor. In addition, radioisotopes may be used to deliver ionizing radiation to a tissue or cell.

Surgery. Approximately 60% of patients with cancer undergo surgery of some type, which includes preventive, diagnostic or staging, curative and palliative surgery. Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised and/or destroyed. Tumor resection refers to physical removel of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically controlled surgery. Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy, such as with the Alu-retrotransposon-based vector of the invention (or a pharmaceutical composition thereof). Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every week for up to 5 or 6 weeks or ever month for up to 12 months. These treatments may be of varying dosages as well.

Chemotherapeutic Agents. In certain embodiments, an Alu-retrotransposon vector according to the present invention, or a pharmaceutical composition thereof, is used in combination with one or more chemotherapeutic agents. Agents that affect DNA function are defined as chemotherapeutic agents, for example, agents that directly cross-link DNA, agents that intercalate into DNA, and agents that lead to chromosomal and mitotic aberrations by affecting nucleic acid synthesis. Some examples of chemotherapeutic agents include, but are not limited to, antibiotic chemotherapeutics such as Doxorubicin, Daunorubicin, Mitomycin (also known as mutamycin and/or mitomycin-C), Actinomycin D (Dactinomycin), Bleomycin, Plicomycin. Plant alkaloids such as Taxol, Vincristine, Vinblastine. Miscellaneous agents such as Cisplatin, VP16, Tumor Necrosis Factor. Alkylating Agents such as, Carmustine, Melphalan (also known as alkeran, L-phenylalanine mustard, phenylalanine mustard, L-PAM, or L-sarcolysin, is a phenylalanine derivative of nitrogen mustard), Cyclophosphamide, Chlorambucil, Busulfan (also known as myleran), Lomustine. other agents comtemplated are for example, Cisplatin (CDDP), Carboplatin, Procarbazine, Mechlorethamine, Camptothecin, Ifosfamide, Nitrosurea, Etoposide (VP16), Tamoxifen, Raloxifene, Estrogen Receptor Binding Agents, Gemcitabien, Navelbine, Farnesyl-protein transferase inhibitors, Transplatinum, 5-Fluorouracil, and Methotrexate, Temazolomide (an aqueous form of DTIC), or any analog or derivative variant of the foregoing.

Immunotherapy. In certain embodiments, an Alu-retrotransposon vactor according to the present invention, or a pharmaceutical composition thereof, is used in combination with one or more immunotherapeutic agents. Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface

molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. A gene encoding a toxic protein when transferred to cancer cells can cause cell death and apoptosis. The apoptotic cancer cells are scavenged by reticuloendothelial cells including dendritic cells and macrophages and presented to the immune system to generate anti-tumor immunity. Immune stimulating molecules may be provided as immune therapy: for example, cytokines such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as FLT3 ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in combination with an Alu-retrotransposon-based vector of the present invention will enhance anti-tumor effects. Immunotherapy may be of the following types: (i) Passive Immunotherapy which includes: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotype antibodies; and finally, purging of tumor cells in bone marrow; and/or (ii) Active Immunotherapy wherein an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant, and/or (iii) Adoptive Immunotherapy wherein the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered.

E. Other Uses

The present invention also provides a method for delivering a gene or nucleic acid sequence of interest in a cell that over-expresses LINE-1 which comprises a step of introducing an Alu-retrotransposon-based vector according to the present invention into the cell. Introducing an Alu-retrotransposon-based vector according to the present invention into the cell may be achieved by *via* transformation, transduction, transfection or infection, or via a lipid, a liposome or a nanoparticle. This method is generally practiced *in vitro*. However, it is contemplated that the method may be used *in vivo*, for example in a biological system, or animal, for example an animal model.

III - Pharmaceutical Compositions

As mentioned above, an Alu-retrotransposon-based vector according to the present invention may be administered *per se* or as a pharmaceutical composition.

Accordingly, the present invention provides pharmaceutical compositions that comprise an effective amount of an Alu-retrotransposon-based vector described herein and at least one pharmaceutically acceptable carrier or excipient. In some embodiments, the pharmaceutical composition further comprises one or more additional biological active agents.

An Alu-retrotransposon-based vector of the invention, or a pharmaceutical composition thereof, may be administered in any amount and using any route of administration effective for achieving the desired therapeutic effect. The optimal pharmaceutical formulation can be varied depending upon the route of administration and desired dosage. Such formulation may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the administered active ingredient.

The pharmaceutical compositions of the present invention may be formulated in dosage unit form for ease of administration and uniformity of dosage. The experession "unit dosage form", as used herein, refers to a physically discrete unit of an inventive construct for the patient to be treated. It will be understood, however, that the total daily dosage of the compositions will be decided by the attending physician within the scope of sound medical judgement.

A. Formulation

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions, may be formulated according to the known art using suitable dispersing or wetting agents, and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension or emulsion in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 2,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solution or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. Fatty acids such as oleic acid may also be used in the preparation of injectable formulations. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration.

Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile

solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use. Liquid pharmaceutical compositions which are sterile solutions or suspensions can be administered by, for example, intravenous, intramuscular, intraperitoneal or subcutaneous injection. Injection may be *via* single push or by gradual infusion. Where necessary or desired, the composition may include a local anesthetic to ease pain at the site of injection.

In order to prolong the effect of an active ingredient (here an Alu-retrotranposon vector), it is often desirable to slow the absorption of the ingredient from subcutaneous or intramuscular injection. Delaying absorption of a parenterally administered active ingredient may be accomplished by dissolving or suspending the ingredient in an oil vehicle. Injectable depot forms are made by forming micro-encapsulated matrices of the active ingredient in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of active ingredient to polymer and the nature of the particular polymer employed, the rate of ingredient release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations can also be prepared by entrapping the active ingredient in liposomes or microemulsions which are compatible with body tissues.

Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, elixirs, and pressurized compositions. In addition to the expression vector according to the invention, the liquid dosage form may contain inert diluents commonly used in the art such as, for example, water or other solvent, solubilising agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cotton seed, ground nut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols, and fatty acid esters of sorbitan and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, suspending agents, preservatives, sweetening, flavouring, and perfuming agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Examples of suitable liquid carriers for oral administration include water (potentially containing additives as above, *e.g.*, cellulose derivatives, such as sodium carboxymethyl cellulose solution), alcohols (including

monohydric alcohols and polyhydric alcohols such as glycols) and their derivatives, and oils (*e.g.*, fractionated coconut oil and arachis oil). For pressurized compositions, the liquid carrier can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Solid dosage forms for oral administration include, for example, capsules, tablets, pills, powders, and granules. In such solid dosage forms, an Alu-retrotransposon-based vector of the invention may be mixed with at least one inert, physiologically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and one or more of: (a) fillers or extenders such as starches, lactose, sucrose, glucose, mannital, and silicic acid; (b) binders such as, for example, carboxymethylcellulose, alginates, gelatine, polyvinylpyrrolidone, sucrose, and acacia; (c) humectants such as glycerol; (d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (e) solution retarding agents such as paraffin; absorption accelerators such as quaternary ammonium compounds; (g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate; (h) absorbents such as kaolin and bentonite clay; and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulphate, and mixtures thereof. Other excipients suitable for solid formulations include surface modifying agents such as non-ionic and anionic surface modifying agents. Representative examples of surface modifying agents include, but are not limited to, poloxamer 188, benzalkonium chloride, calcium stearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatine capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition such that they release the active ingredient(s) only, or preferably, in a certain part of the intestinal tract, optionally, in a delaying manner.

Examples of embedding compositions which can be used include polymeric substances and waxes.

In certain embodiments, it may be desirable to administer an inventive composition locally to an area in need of treatment. This may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, by injection, by means of a catheter, by means of suppository, or by means of a skin patch or stent or other implant.

For topical administration, the composition is preferably formulated as a gel, an ointment, a lotion, or a cream which can include carriers such as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oil. Other topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylenemonolaurat (5%) in water, or sodium lauryl sulphate (5%) in water. Other materials such as antioxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary.

In addition, in certain instances, it is expected that the inventive compositions may be disposed within transdermal devices placed upon, in, or under the skin. Such devices include patches, implants, and injections which release the active ingredient by either passive or active release mechanisms. Transdermal administrations include all administrations across the surface of the body and the inner linings of bodily passage including epithelial and mucosal tissues. Such administrations may be carried out using the present compositions in lotions, creams, foams, patches, suspensions, solutions, and suppositories (rectal and vaginal).

Transdermal administration may be accomplished through the use of a transdermal patch containing an active ingredient (*i.e.*, an Alu-retrotransposon-based vector according to the invention) and a carrier that is non-toxic to the skin, and allows the delivery of the ingredient for systemic absorption into the bloodstream *via* the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may be suitable. A variety of occlusive devices may be used to release the active ingredient into the bloodstream such as a semi-permeable membrane covering a

reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient.

Suppository formulations may be made from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerine. Water soluble suppository bases, such as polyethylene glycols of various molecular weights, may also be used.

A pharmaceutical composition according to the present invention may further comprise at least one transfection reagent. Transfection reagents commonly known to one skilled in the art that may be employed include, but are not limited to, cationic lipid transfection reagents, cationic lipid mixtures, polyamine reagents, liposomes and combinations thereof; SUPERFECT™, Cytofectene, BioPORTER™, GenePORTER™, NeuroPORTER™, and perfectin from Gene Therapy Systems; lipofectamine, cellfectin, DMRIE-C oligofectamine, and PLUS reagent from InVitrogen; Xtreme gene, fugene, DOSPER and DOTAP from Roche; Lipotaxi and Genejammer from Strategene; and Escort from SIGMA. As known in the art, the ratio of DNA to transfection reagent may vary based upon the method of administration.

Materials and methods for producing various formulations are known in the art and may be adapted for practicing the present invention. Suitable formulations can be found, for example, in "*Remington's Pharmaceutical Sciences*", E.W. Martin, 18th Ed., 1990, Mack Publishing Co.: Easton, PA.

B. Additional Biologically Active Agents

In certain embodiments, an Alu-retrotransposon-based vector is the only active ingredient in a pharmaceutical composition of the present invention. In other embodiments, the pharmaceutical composition further comprises one or more biologically active agents. Examples of suitable biologically active agents include, but are not limited to, therapeutic agents such as anti-cancer agents (as described above), anti-inflammatory agents, immunomodulatory agents, analgesics, antimicrobial agents, antibacterial agents, antibiotics, antioxidants, antiseptic agents, and combinations thereof.

In the pharmaceutical compositions of the present invention, the Alu-retrotransposon-based vector and additional therapeutic agent(s) may be combined in

one or more preparations for simultaneous, separate or sequential administration of the Alu-retrotransposon-based vector and the therapeutic agent(s). More specifically an inventive composition may be formulated in such a way that the Alu-retrotransposon-based vector and therapeutic agent(s) can be administered together or independently from each other. For example, the Alu-retrotransposon-based vector and therapeutic agent can be formulated together in a single composition. Alternatively, they may be maintained (*e.g.*, in different compositions and/or containers) and administered separately.

C. Pharmaceutical Packs of Kits

In another aspect, the present invention provides a pharmaceutical pack or kit comprising one or more containers (*e.g.*, vials, ampoules, test tubes, flasks or bottles) containing one or more ingredients of an inventive pharmaceutical composition, allowing administration of an Alu-retrotransposon-based vector of the present invention.

Different ingredients of a pharmaceutical pack or kit may be supplied in a solid (*e.g.*, lyophilized) or liquid form. Each ingredient will generally be suitable as aliquoted in its respective container or provided in a concentrated form. Pharmaceutical packs may include media for the reconstitution of lyophilized ingredients. Individual containers of the kits will preferably be maintained in close confinement for commercial sale.

The kits of the invention may also comprise, or be packaged with, an instrument for assisting with the injection/administration of the Alu-retrotransposon-based vector, or pharmaceutical composition thereof, or with the placement of the Alu-retrotransposon-based vector, or pharmaceutical composition thereof, within the body. Such an instrument may be a syringe, pipette, forceps, or any such medically approved delivery device.

In certain embodiments, a pharmaceutical pack or kit includes one or more additional therapeutic agent(s) (*e.g.*, one or more anti-cancer agents, as described above). Optionally associated with the container(s) can be a notice or package insert in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical or biological products, which notice reflects approval by the agency

of manufacture, use or sale for human administration. The notice of package insert may contain instructions for use of a pharmaceutical composition according to methods of treatment disclosed herein.

An identifier, *e.g.*, a bar code, radio frequency, ID tags, etc., may be present in or on the kit. The identifier can be used, for example, to uniquely identify the kit for purposes of quality control, inventory control, tracking movement between workstations, etc.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

Examples

The following examples describe some of the preferred modes of making and practicing the present invention. However, it should be understood that the examples are for illustrative purposes only and are not meant to limit the scope of the invention. Furthermore, unless the description in an Example is presented in the past tense, the text, like the rest of the specification, is not intended to suggest that experiments were actually performed or data were actually obtained.

Example 1: Overexpression of LINE-1 in tumor cells

Materials and Methods

Lu1205 cells were grown in MW 489 (1/4 LEIBOVITZ + 3/4 MCDB 153) supplemented with 4% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin and L-Glutamine 2 mM (all from Life Technologies, Inc.). Cells were maintained in a 7% CO_2-humidified incubator at 37°C. Mel501 cells were grown in RPMI without HEPES supplemented with 10% FCS, humidified incubation in 5% CO_2 at 37°C. Hermes 3A and Hermes 1 cells were grown in RPMI 1640 supplemented with phenol red 7.5 µg/ml,10% FCS, TPA (Tetradecanoylphorbol-13-acetate) 200 nM, Cholera Toxin 200 pM, hSCF 10 ng/ml, EDN-1(Endothelin-1) 10 nM, penicilline 100 UI/ml, streptomycine 100 µg/ml and glutamine 2 mM. Incubation in 10% CO_2. MCF7 cells were grown in DMEM (1g/l glucose) supplemented with 10% FCS, insuline 0.6 µg/ml and gentamicine 40 µg/ml. Incubation in 5% CO_2. T47D cells were

grown in RPMI 1640 without HEPES supplemented with 10% FCS, insuline 0.6 µg/ml and gentamicine 40 µg/ml. Incubation in 5% CO_2. MCF10A cells were grown in DMEM(1g/L Glucose) / HAM F12 (3:1) supplemented with adenine 20µg/ml, insuline 5 µg/ml, apo-Transferrine human 5 µg/ml, tri-iodothyronine 1.5 ng/ml, hEGF 2 ng/ml, hydrocortisone 0.5 µg/ml, 10% FCS and gentamicine 40 µg/ml. Incubation in 5% CO_2. C19, Calu-1 and Calu-2 cells were grown in alpha 1900 supplemented with 10%FCS and gentamicine 40µg/ml. Incubation in 5% CO_2. A549 cells were grown in DMEM (1g/l Glucose) / HAM F12 (1:1) supplemented with 10%FCS and gentamicine 40µg/ml. Incubation in 5% CO_2. Wi-38 cells were grown in MEM (INVITROGEN) supplemented with 10% FCS, AANE 0.1mM, sodium pyruvate 1 mM and gentamicine 40 µg/ml. Incubation in 5% CO_2. Caco2 cells were grown in DMEM (3.7 g/l NaHCO3) supplemented with 20%FCS, AANE and gentamicine 40µg/ml. Incubation in 10% CO_2. HT29, Colo320 cells were grown in RPMI w/o HEPES supplemented with 10% FCS, without gentamicine. Incubation in 5% CO_2. Sw-620 cells were grown in Leibovitz's L-15 medium supplemented with 10% FCS and gentamicine 40 µg/ml. Incubation in 0% CO_2. A172 cells were grown in DMEM (4.5g/l glucose) supllemented with 10 %FCS and gentamicine 40µg/ml. Incubation in 10% CO_2. U-87 cells were grown in MEM supplemented with 10% FCS, AANE 0,1 mM and sodium pyruvate 1mM. Incubation in 5% CO_2.

RNA isolation and Real-time qPCR analysis. RNA was extracted from each cell line cultured in P10 dishs by using RNeasy extraction columns (Macherey-Nagel) according to the manufacturer's instructions. RNA was treated with 10 U of RNAse-free DNase RQ1 (Roche) per μg RNA for 120 minutes at 37 °C in appropriate buffer. DNAse was inactivated by incubation for 10 minutes at 65 °C. Total RNA (0.5 µg) was reverse transcribed (RT) with superscript II reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. Quantitative PCR was done using the SYBR master mix kit (Roche) for the genes of interest (ORF1, ORF2) and reference gene (β-actin). Primers used are as follows:

ORF1 forward primer: SEQ ID NO: 6: AAAGGGAAGCCCATCAGACT

reverse primer : SEQ ID NO: 7: GCCTGGTGGTGACAAAATCT

ORF2 forword primer: SEQ ID NO: 8: AAAGCAATGGCAACAAAAGC

reverse primer : SEQ ID NO: 9: CCACTTTTTGATGGGGTTGT

β-Actin forward primer : SEQ ID NO: 10: GGCATCCTCACCCTGAAGTA

reverse primer : SEQ ID NO: 11: GGGGTGTTGAAGGTCTCAAA.

Plates were run on the LightCycler 480 (Roche, Vilvoorde, Belgium). The average threshold cycle of triplicate reactions was used for all subsequent calculations using the deltaCT method. Graphs represent the average normalized relative expression values of ORF1 and ORF2.

Results

Real-time analysis of LINE-1 expression in several human cancer cell lines has been performed. The results presented in Figure 2 show significantly higher expression of both LINE-1 transcripts (ORF1 and ORF2) in cancer cell lines that in corresponding control healthy cell lines. Moreover, the level of ORF1 and ORF2 expression was found to be similar in almost all cancer cell lines tested.

Transcripts (mRNAs) extracted from cancer tissues or corresponding control tissues have been directly tested. Eleven types of tissues have been tested (prostate, colon, ovary, skin, breast, liver, pancreas, testicle, stomach, kidney, bone). For different reasons (such as quality of some samples, problems with RNA normalization, lack of ideal control healthy tissue samples), the preliminary results obtained have only allowed the identification of two cancer tissues (prostate and testicle) for which the expression levels of ORF1 and ORF2 were significantly higher than those measured in available control healthy samples. This confirms that some cancers exhibit a LINE-1 activity that is higher than in corresponding healthy tissue samples.

Example 2: Construction of the Alu-HSV-TK_mClover Vector with the self-splicing Tet Intron and Validation of Integration

Materials and Methods

Production of polyclonal anti HSV-TK antibody. The gene coding for HSV-TK was cloned into the pET28 *E.coli* expression vector. The expression vector carrying (His)6-HSV-TK was transformed into *E.coli* DE3 strain. The culture was induced with 1 mM IPTG at an OD_{600} for 4 hours at 37°C. The protein was purified using the Ni-NTA affinity chromatography. *E. coli* BL21 (DE3) over-expressing *HSV-TK* were

lysed by sonication. The resuspension buffer, 50 mM Tris-Cl, pH 8.0; 150 mM NaCl; 15 mM imidazole, was supplemented with 1 mM PMSF (phenyl methyl sulfonyl fluoride). Upon centrifugation of the cell lysate, the supernatant obtained was loaded onto the Ni-NTA column equilibrated with the resuspension buffer. The column was washed with 8-10 bed volumes of the same buffer. Protein was eluted with 50 mM Tris-Cl, pH 8.0; 150mM NaCl; l00 mM imidazole. The recombinant protein then used to immunize the rabbit. The antiserum was collected and tested.

Immunostaining. Hek293T cells were seeded onto cover-slips in a cell culture dish 96 hours after transfection with A2 or cotransfection with HSV-TK and GFP expression vectors as positive control. Experiments were terminated by fixing cells with 4% paraformaldehyde for 10 minutes. Fixed cells were washed with PBS and permeabilized with 1% Triton X-100 in PBS for 15 minutes. After washing with PBS, cells were incubated with antiserum anti HSV-TK (1:2000 dilution) at 4°C overnight, then incubated with Alexa Fluor 555 conjugated-anti-rabbit IgG (1: 500 dilution; Invitrogene) for 1 hour, and stained nuclei with DAPI (Invitrogen, Life Technologies, Grand Island) for 2 minutes. The slides were embedded in Vectashield. Images of cells with fluorescent signal were captured by laser confocal microscopy (Leica Sp5 Laser Scanning Confocal Microscope, GE).

Plasmids. The Alu-neo^{Tet} vector was kindly provided by Heidmann T. The construct was modified as follows: the inventors inserted the antisenese mClover fused to HSV-YK (the antisense stop codon of the HSV1-TK was removed and an anti sequence encoding 11 amino acid linker (SEQ ID NO: 12: LEU ARG ASP PRO MET ALA ARG ALA ALA ALA THR) was added in-frame between the TK and GFP open reading frames. The HSV-TK-GFP was interrupted by a tet intron. The tet intron position was chosen in order to conserve IGS base pairing as possible according to (Esnault *et al.* NAR 2002, 30, No 11 e49). A poly A (50 bases) according to (Matthew *et al.*, Genome Research, 2009, 19:545-555) was introduced upstream antisense SV40 promoter. A transcription terminator of TTTTT motif was fused downstream of the Alu poly-A tract.

Results

HSV-TK Transposition through Alu-L1 Canonical Retrotransposition Process.

To assay for HSV-TK retrotransposition cycle, which includes transcription, reverse

transcription and integration (Figure 3(A)), the vector designed and developed here consists in a pU57_simple backbone with an AluYa5 sequence driving the transcription of a retrotransposition RNA intermediate comprising a bright monomer GFP (Shaner *et al.* Nature Methods, 2013, 10: 407–409, doi:10.1038/nmeth.2413) as a gene marker, the HSV-tk gene inactivated by the presence of a Tetrahymena self-splicing intron and a SV40 promoter (This construct will also be called the A2 construct).

To validate the expression of HSV-TK protein, the inventors generated a polyclonal antibody against HSV-TK and have used this antibody to verify the presence of HSV-TK in cells that cotransfected with HSV-TK and GFP expression vectors (Figure 3B upper panel). It was observed that some cells have both HSV-TK signals and other cells have only one signal due to the transfection variety. However, these results indicate that the antibody works in immunostaining. Then HEK393 cells were transfected with A2 construct. 96 hours after transfection, they looked for HSV-TK by immunoblotting, and GFP signal endougenous. The results show (Figure 3A bottom panel) colocalisation of HSV-TK/GFP signal indicating that A2 constructs enable the expression of the HSV-TK fused to GFP protein in some of the transfected cells.

Example 3: HSV-TK genome integration

Materials and Methods

DNA FISH. Labelled A2 probe was used for DNA-FISH. First, A2 was labelled with atto 647 by nick translation, 500 ng of A2 plasmid mixed with labelled dNTPs and nick translation mix (Roche) all incubated at 15°C for 90 minutes and then heat inactivation at 75°C for 10 minutes. Slides with HEK 293 cells were pretreated with RNaseA. The genomic DNA was denatured by incubating them for 90 seconds at 80°C in 60% formamide, 2 x SSC, pH 7 on a hotplate. After removal of the coverslip, the slides were dehydrated through an ethanol series and air dried. Then, 10 µl hybridization mixture was applied under a 18 x 18 mm coverslip, sealed with rubber cement, and hybridisation was performed overnight at 37°C in a humid chamber. The hybridization mixture contained 50% formamide, 2xSSC, 50 mM sodium phosphate pH7, 10% dextran sulphate, 300 ng of the A2 probe, 3 x excess human Cotl-DNA and 10 x excess low molecular weight fish sperm DNA in 10 µl. Before application, the probes were denatured for 10 minutes at 80°C, followed by 60 minutes incubation at 37°C to allow pre-annealing with the 3 x excess of Cotl-DNA.

After a 10 min post-hybridization wash in 2 x SSC/0.1% Tween 20 at 37°C to remove the coverslips, the slides were washed 2 x 5 min in 50% formamide, 2 x SSC, pH 7 at 44°C. This was followed by 2 washes (5 min each) in 0.1 x SSC at 60°C and a 5 min wash at RT in TNT (0.1M Tris HCl pH7.4, 0.15 M NaCl, 0.05% Tween 20). Chromosomes were counterstained with DAPI. The slides were embedded in Vectashield. Images of cells with fluorescent signal were captured by laser confocal microscopy (Leica Sp5 Laser Scanning Confocal Microscope, GE).

Results

To test if the A2 construct was effectively inserted within the genome after the transfection, the vector integration has been directly visualised by FISH. The results clearly show that A2 was integrated within the host cells genome. Interestingly, this integration took place, in some cases, at several luci in the same cell, whereas in some cells the integration did not take place.

These data provide evidence that the retro-transposition mechanism is active in cells expressing L1 proteins.

Example 4: Efficiency *in vitro* Tests

Materials and Methods

Cell Death Assays. Cell lines used were MCF-7, HT-29, A172, U87, and WI-38. Cells (5.10^5) were seeded into a p10 dish and grown in a corresponding medium d0. At d0 cells were transfected with (A2, mClover), transfection was carried out using (FuGENE, Promega), plasmids used for transfection were 20 µg per dish, ratio DNA/FuGENE was (1/6). 36 hours after transfection (d3), the cells were traited with Ganciclovir (GCV) (Sigma-Aldrich) at 10 µg/ml for 7 days (after 3 days of traitement (d6) medium was changed with fresh GCV). Cell viability was measured at d11. Briefly, cells were collected, washed 2X in PBS before re-suspension in binding buffer and then 2µg/mL propidium iodide (PI) and APC congugated Annexin V (BD Biosciences) were added. Percentages of labelled cells were determined by flow cytometry (BD FACSCalibur). All experiments were performed at least twice and average is presented. The data are expressed as mean ± s.d. for triplicate. Inducing apoptosis was achieved using Etoposide (Sigma-Aldrich) at 10 µg/ml for 48 hours.

Western Blotting. Whole cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 minutes, the membrane was washed once with TBST and incubated with antibodies against ORF1 (1:1000) or tubulin (1:5000) at 4 °C for 12 hours. Membranes were washed three times for 10 minutes and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies for 2 hours. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols.

Results

Combining the ORF1p and ORF2p expression data obtained on cell lines with experimental contingencies (like cell culture and cell transfection constrains), the *in vitro* proof of concept was achieved using the breast cancer cell line MCF7 and the colon cancer cell line HT-29.

Different control cell lines were chosen to assess the selectivity of the strategy:

- The WI-38 cell line is a non-tumorigenic cell line from ectodermal origin. This cell line displayed a low level of ORF1 and ORF2 mRNA expression.
- The A172 and U87 are both cell lines from glioblastoma cancer. They were selected based on recently published results (Lee *et al.*, Science, 2012, 337(6097): 967-971). In this study, the authors performed single-nucleotide resolution analysis of transposable elements insertion in whole-genome sequencing data sets from five cancer types. The results show somatic insertion of L1 and Alu in different tumors of epithelial origin but not in blood or brain cancers.

All these selected cell lines have been transfected with the A2 construct, or with a GFP control expression vector, followed or not by a treatment with the pro drug Ganciclovir (GCV). Seven days after the treatment of the transfected cells with GCV, the induced cell death was measured by Fluorescence-activated cell sorting (FACS), using Annexin V and propidium iodure as markers of apoptosis and necrosis respectively.

These data show that the cell death observed for the MCF7 and HT-29 cell lines is at least doubled after GCV treatment, when the A2 vector is transfected and the HSV-

TK enzyme expressed, as compared to a transfection with a GFP expression vector and GCV treatment or to a transfection with the A2 vector without GCV treatment. It is important to note that the transfection efficiency was variable for each experimental condition and was never higher than 10% of the total cells. Therefore, the proportion of the cells potentially expressing HSV-TK could never exceed 10% of the cells analyzed in these experiments.

Of note also, although the melanoma Mel501 cells express ORF1p, preliminary cell death induction experiments could not show sensitivity to the A2 transfection. As a control positive of apoptosis induction, traitement with Etoposide was sufficient to induce apoptosis in A172 and U87 showing that apoptosis could be triggered in these cell lines. In order to link the activity observed by FACS analysis into ORF machenry, the inventors tested the level of ORF1 protein in the tested cell lines. Interestingly, ORF1 protein was very abundant in MCF7 and HT-29 where FACS analysis showed apoptosis induction.

The results obtained here demonstrate the efficacy of the SINE-directed suicide gene strategy when Alu-HSV-TK vector is transfected in cancer cell lines expressing the L1 ORF1 protein. The absence of increased cell death in the different control cell lines could also provide a good indication on the selectivity and harmlessness of this approach towards cells that do not have L1 activity.

Example 5: Absence of ORF1 in Hela Cells

Materials and Methods

Cell extraction. Cells were grown in P10 dish till 80% confluence. The cell culture dish was placed on ice and the cells were washed with ice-cold PBS. The PBS was drained off, then 1 mL of ice-cold lysis buffer (10 mM Tris, pH 7.4 500 mM KCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol) was added to each dish. Adherent cells were scraped off the dish using a cold plastic cell scraper, then the cell suspension obtained was transfered into a pre-cooled microfuge tube, and submitted to a 3x cycling of liquid nitrogen freezing and 37°C thawing. The cells were maintained on ice for 30 minutes, then centrifuged in a microcentrifuge at 4°C at 12 krpm for 30 minutes. The pellet obtained was discarded and the supernatant was used for analysis.

Western Blotting. The protocole described in Example 4 above was used.

Results

The results obtained (see Figure 6) show that the ORF1 protein is not expressed in all cancer cell lines even though the level of RNA is relatively high indicating a post transcription regulation of ORF1 protein level. This could explain the rare L1-mediated Alu retrotransposition events in the experiments carried out by Dewannieux *et al.* ("LINE-mediated retrotransposition of marked Alu sequences", Nature Genetics, 2003, 35(1): 41-48), where the authors proved the dependence of Alu on L1 ORF for its retrotransposition.

On the other hand, the absence of ORF1 protein in Hela cells shows the importance of ORF screening over cancer cell lines to identify the potential target of the present invention.

Example 6: Increase of Apoptosis in MCF-7 and HCT116 Cells

Materials and Methods

At Day 0, cells (MCF-7, HCT116) were seeded into 6-well plates and grown in a corresponding medium. Transfection was performed on the same day with HSV-tk (A2) and GFP vectors. In the case of MCF7 cells, transfection was carried out using FuGENE (Promega). Four (4) µg of plasmid were used for transfection per well with a ratio DNA/FuGENE of 1/6. In the case of the HCT116 cells, transfection was carried out using Lipofectamine 2000 (Invitrogen), in the same quantity and transfection ratio as above. The medium was changed 6 hours after transfection. Twenty-four (24) hours after transfection, MCF7 cells were transfected a second time in the same manner as described above. At Day 3 (*i.e.*, 36 hours after the first transfection), the cells were treated with Ganciclovir (GCV – Sigma-Aldrich) at 10 µg/mL for 7 days. At Day 6 (*i.e.*, after 3 days of treatment), the medium was changed with fresh Ganciclovir.

Cell viability was measured at Day 11. Briefly, cells were collected, washed twice in PBS before being re-suspending in buffer and then adding 2 g/mL propidium iodide (PI) and APC conjugated Annexin V (BD Biosciences). The percentage of labelled cells was determined by flow cytometry (BD FACS Calibur). All the experiments were performed at least twice and the average is presented. The data are expressed as mean ± s.d. for triplicate. Apoptosis was induced using Etoposide (Sigma-Aldrich) at a concentration of 10 µg/mL for 48 hours.

Results

The results obtained (see Figures 7 and 8) confirmed the efficiency of the present invention on the MCF7 cell line putting the breast cancer in the first line as target for the therapy described herein. Furthermore, the results obtained show that theHCT116 cell line responds to the GCV treatment indicating the potential of the inventive strategy for colon cancer as well.

Example 7: ORF1 Protein Expression Screening in Cancer Cell Lines

Materials and Methods

Cell extraction. The protocol described in Example 5 was used.

Western Blotting. The protocole described in Example 4 above was used.

Results

The results obtained (see Figure 9) show that ORF1 is expressed differently in different cancer cell lines. In the ScaBER bladder cell line, ORF1 was found to have a strong expression level. In ovary cell lines, the ORF1 expression was restricted to oncogenic transformation. Indeed, the Caov3 cancer cell line was found to express ORF1n whereas in the SVG40 normal cell line, there was no ORF1 expression. The same was observed in lung cell lines (no ORF1 expression occurred in the IMR90 normal lung cell line, whereas the NCI-H1975, Calu6 and NCI-H1435 lung cancer cell lines expressed ORF1). The pharynx celle lines showed high levels of ORF1, the stomach cell line also showed the presence of ORF1 protein, but no ORF1 expression was observed in the in tested pancreatic cell lines.

Taking all together, these results suggest that the proposed therapy could be efficient for bladder, ovary, lung, stomach, and pharynx cancers.

It is worth noting that the present screening could be extended to other type of cancers and it will be very important to test the efficiency *in vitro* and *in vivo*.

Example 8: Construction of an AAV Vector

In orther to achieve better cell targeting efficiency and to have a therapeutic vehicle for *in vivo* testing, the Alu-HSVètk construct tested *in vitro*, was cloned into an adeno-associated virus (AAV) vector. Since AAV vectors do not integrate into the genome, they are diluted from dividing cells. This feature is very useful in the present

projet since the Inventors select for L1 driven integration events, as opposed to random integration of the vector. The use of an AVV approach has a number of features that make AVV an ideal vector for gene therapy strategies: it offers long-term gene expression, there is little to no cell-mediated immune response to the virus, and it is not associated with any disease. The suitable of AAV for tumor treatment lies in its ability to efficiently and stably transfect a wide range of cells, including dividing and non-dividing cells. It also has the abilty to penetrate the stroma of solid tumors due to its small size and can offer an excellent safety profile combined with reduced potential for activation of inflammatory or cellular immune responses.

Numerous AAV serotypes have been identified with variable tropism; however all of them share some properties including genome size and organization and, with small differences, the inverted terminal repeats share structure and function. The ability to transduce different cell types is primarily determined by the AAV protein capsid. The different capsids bind to different cellular receptors and this binding mediates entry into the cell.

As a starting point, the Inventors decided to use AAV-DJ recombinant serotype (Cell Biolabs, Inc). This serotype has been selected over a complex library of hybrid capsids from eight different wild-type viruses. Stringent selection of AAV variants on human liver cells and with human anti-AAV antisera result in AAV-DJ, and AAV-DJ/8 (a heparin binding domain mutant of AAV-DJ). Recombinant AAV-DJ vectors mediate superior *in vitro* transduction efficacies in comparison with any other wild type serotypes. Transduction on cell types from different species and tissues, including primary human hepatocytes, melanoma cells, and embryonic stem cells, showed that AAV-DJ vectors were not only superior to all HBD-negative wild-type viruses (up to 100,000-fold better than AAV-8 or AAV-9), but also substantially better than AAV-2. The heparin binding domain (HBD) plays important role for *in vivo* viral infection as demonstrated by comparing AAV-DJ to the DJ/8 mutant: HBD deletion alleviated the liver restriction and expanded transduction to all nonhepatic tissues, including the brain,

Materials and Methods

A2 Cloning in pAAV. A2 was cloined in pAAV vector using a standard restriction cloning protocol.

rAAV Production and rAAV Primary Stock Preparation.

Cells. AAV-293 cells (Stratagene #240073) were used. AAV-293 cells are derived from the commonly used HEK293 cell line, but produce higher viral titers. HEK293 cells are human embryonic kidney cells that have been transformed by sheared adenovirus type 5 DNA. AAV-293 cells, like HEK293 cells, produce the adenovirus E1 gene in *trans,* allowing the production of infectious adeno-associated virus particles when they are co-transfected with the three AAV Helper-Free System plasmids (an ITR-containing plasmid, pAAV-RC, and the E1-deleted pHelper plasmid). The cells were cultured in DMEM (4,5g/L glucose) with GLUTAMAX-1 supplemented with 10% FCS and gentamycin. When the cell monolayer reached 50% confluence, they were diluted 1/5-1/6 every 2 days or 1/15 over the week-end.

Plasmids. pHelper plasmid supplies adenovirus helper genes E2, E4 and VA RNA genes pAAV-RC, pXR1, pXR5, pAAV2/8, pAAV2/9, pAAV2/rh10 plasmids supply replication and capsid genes of serotypes 2, 1, 5, 8, 9 & 10. pAAV-X plasmid supplies the transgene expression cassette flanked by the AAV ITR2s.

rAAV Production in Culture Plates. One day (D-1) before co-transfection, 8 to $10x10^6$ cells were platted per 150 mm-tissue culture plate in 25 mL of DMEM growth medium. The plate was gently swirled 10x for a homogeneous distribution of cells and returned to the incubator. On Day 0, the cells were transfected using X-tremeGENE 9 DNA transfection reagent. The cells should be ~50-60% confluent. Plasmids were mixed at equimolar ratio: 120 µg pHelper, and 60 µg pAAV-RC or any plasmid containing AAV replication and AAV capsid genes, 40-60 µg of the recombinant pAAV expression plasmid. 15 mL of OptiMEM were pipetted into a 50 mL tube, to which 600 µL X-tremeGENE 9 DNA Transfection reagent were quickly added. The mixture was vortexed for a short time and incubated 5 minutes at room temperature. It was then added dropwise into the the plasmid mix. The mixture obtained was vortexed for a short time and incubated for 20 minutes at room temperature, and then poured directly into 250 mL of prewarmed culture medium supplemented with 2M FCS and mixed. 25 mL of this mixture were poured into a 150mmm culture plate. The plates were returned to the 37°C incubator for an additional 48 hours.

Virus production was monitored by checking the phenotype changes to the cell culture (using a negative control, *i.e.*, no-DNA transfection): color change in the

medium from red to orange or yellow, and cells that round up, detach from the plate and can be seen floating in the medium).

Preparation of the rAAV Primary Stock. Viral particles are present in both intact cells and the growth medium. However, the following protocol describes the preparation of a primary viral stock from cell lysate. The cell lysate was prepared as follows. The transfected cells were scrapped and the cell suspension obtained was transferred into 10x50 mL-conical tube. The tube was centrifuged at 1800 rpm for 10 minutes. The cell pellet was resuspended in 24 mL of Iodixanol lysis buffer. The cell suspension obtained was distributed into four 15 mL-conical tubes. The cell suspension was subjected to 3 rounds of freeze/thaw by alternating the tubes between a dry ice-ethanol bath and a 37°C water bath, vortexing after each thaw. The nuclease treatment was carried out as follows. 0.4 mL of Benzonase (10 U) was added to each mL of lysate and the mixture was incubated for 1 hour at 37°C (and mixed every 10 minutes). The cellular debris were pelleted at 3500 rpm for 15 minutes. The supernatant was filtered (lysate and primary virus stock) on 0.45 µm or 5.0 µm filters and transferred to a fresh tube. The viral stocks were stored at -20°C for several weeks or at -80°C for more than one year.

The viral titer was determined by transduction of AAV-HT 1080 cells or Hela cells (TU: transduction Unit/mL) and by Q-PCR (GU: Genomic Unit/mL).

Results

Infection efficiency of DJ serotype in MCF7, HCT16 and HT29. Cells were seeded at $2x10^5$ cells/well in a 6-well plate. The next day, the cells were infected with pAAV-eGFP-DJ virus at several dilutions (2 plates for each dilution). 48 hours after, the cells were prepared for FACS analysis (washed using PBS, trypsinized, neutralized, and resuspended in PBS and then FACA). The results obtained showed that the maximum of positive cells were 84.9%, 93n70% and 95.15% for MCF7, HCT116 and HT29, respectively using $40x10^8$ PV (particle viral), $8x10^8$ PV and $40x10^8$ PV for MCF7, HCT116 and HT29 respectively.

Efficiency of GCV treatment on pAAV-A2 infected cells MCF7. Cells ($2x10^5$) were seeded into a 6-well plate and grown in a corresponding medium on Day 0. At D1, cells were infected with pAAV-A2-DJ using the same PV of pAAV-eGFP-

DJ corresponding to 85% of positively infected cells. 36 hours after infection (D3), the cells were treated with Ganciclovir (GCV) (Sigma-Aldrich) at 10 µg/ml for 7 days (after 3 days of treatment (D6), the medium was changed with fresh GCV). Cell viability was measured at D11. Briefly, cells were collected, washed 2X in PBS before re-suspension in binding buffer and then 2µg/mL propidium iodide (PI) and APC conjugated Annexin V (BD Biosciences) were added. Percentages of labelled cells were determined by flow cytometry (BD FACSCalibur). All experiments were performed at least twice. Induction of apoptosis was achieved using Etoposide (Sigma-Aldrich) at 10 µg/ml for 48 hours.

The results obtained showed that MCF7 infected with pAAV-A2-DJ exhibited three times more of early apoptotic cells than control cells. This indicates that mortality was obviously more important by increasing the percentage of positively infected MCF7, in other words by increasing the percentage of A2 delivery into MCF7, comparing to transfection efficiency about 65% of positive cells, it was possible to induce more cell mortality.

Other Embodiments

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of the specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope of the invention being indicated by the following claims.

Claims

What is claimed is:

1. An expression construct or vector for use in gene therapy, wherein the expression construct or vector comprises an Alu retrotransposon flanked with a gene or nucleic acid sequence and wherein the gene or nucleic acid sequence is to be delivered to a cell which overexpresses LINE-1.

2. An expression construct or vector for use in gene therapy for the treatment of cancer in a subject, wherein the expression construct or vector comprises an Alu retrotransposon flanked with a gene or nucleic acid sequence, and wherein the cancer to be treated over-expresses LINE-1.

3. The expression construct or vector for the use according to claim 2, wherein the cancer to be treated over-expresses LINE-1 is selected in the group consisting of breast cancers, bladder cancers, ovary cancers, lung cancers, stomach cancers, colon cancers, and pharynx cancers.

4. The expression construct or vector for the use according to any one of claims 1 to 3, wherein the expression construct or vector further comprises regulatory sequences operably linked to the Alu retrotransposon and/or to the gene or nucleic acid sequence.

5. The expression construct or vector for the use according to any one of claims 1 to 4, wherein the Alu retrotransposon belongs to the AluY subfamily of retrotransposons, preferably to the AluYa8 subfamily or to the AluYa5 subfamily.

6. The expression construct or vector for the use according to claim 5, wherein the Alu retrotransposon has the sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 2 or has a sequence that is at least 80% homologous to SEQ ID NO: 1 or SEQ ID NO: 2.

7. The expression construct or vector for the use according to any one of claims 1 to 6, wherein the gene is a suicide gene.

8. The expression construct or vector for the use according to any one of claims 1 to 6, wherein the gene is selected from the group consisting of genes that encode a tumor suppressor, genes that encode an inhibitor of apoptosis, genes

that encode a toxin, genes that encode a cytokine and cell cycle regulatory genes.

9. The expression construct or vector for the use according to any one of claims 1 to 6, wherein the nucleic acid sequence is a RNA interfering (RNAi) agent.

10. The expression construct or vector for the use according to any one of claims 1 to 9, wherein the expression construct or vector comprises a plasmid vector or a viral vector, or wherein the expression construct or vector is administered in nanoparticles.

11. The expression construct or vector for the use according to claim 10, wherein the viral vector is selected from the group consisting of retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-associated viral vectors.

12. A pharmaceutical composition for use in gene therapy, preferably for the treatment of cancer in a subject, wherein the pharmaceutical composition comprises a therapeutically effective amount of an expression construct or vector as defined in any one of claims 1 to 11 and at least one physiologically acceptable carrier or excipient.

13. Use of an expression construct or vector as defined in any one of claims 1 to 11 or of a pharmaceutical composition as defined in claim 12 for delivering a gene or nucleic acid sequence to a cell that over-expresses LINE-1.

14. A method for delivering a gene or nucleic acid sequence to a cell that over-expresses LINE-1, wherein the method comprises introducing an expression construct or vector as defined in any one of claims 1 to 11 into the cell.

15. The method according to claim 14, wherein the introducing is achieved *via* transformation, transduction, transfection or infection, or *via* a lipid, a liposome or a nanoparticle.

16. A gene therapy method for treating a cancer in a subject, wherein said method comprises a step of: administering to said subject a therapeutically effective amount of an expression construct or vector as defined in any one of claims 1-2 and 4 to 11, or a pharmaceutical composition thereof.

17. The gene therapy method according to claim 16, wherein the cancer to be treated over-expresses LINE-1.

18. The gene therapy method according to claim 17, further comprising a step of: determining that the cancer to be treated overexpresses LINE-1.

19. The gene therapy method according to claim 17, wherein the cancer that over-expresses LINE-1 is selected in the group consisting of breast cancers, bladder cancers, ovary cancers, lung cancers, stomach cancers, colon cancers, and pharynx cancers.

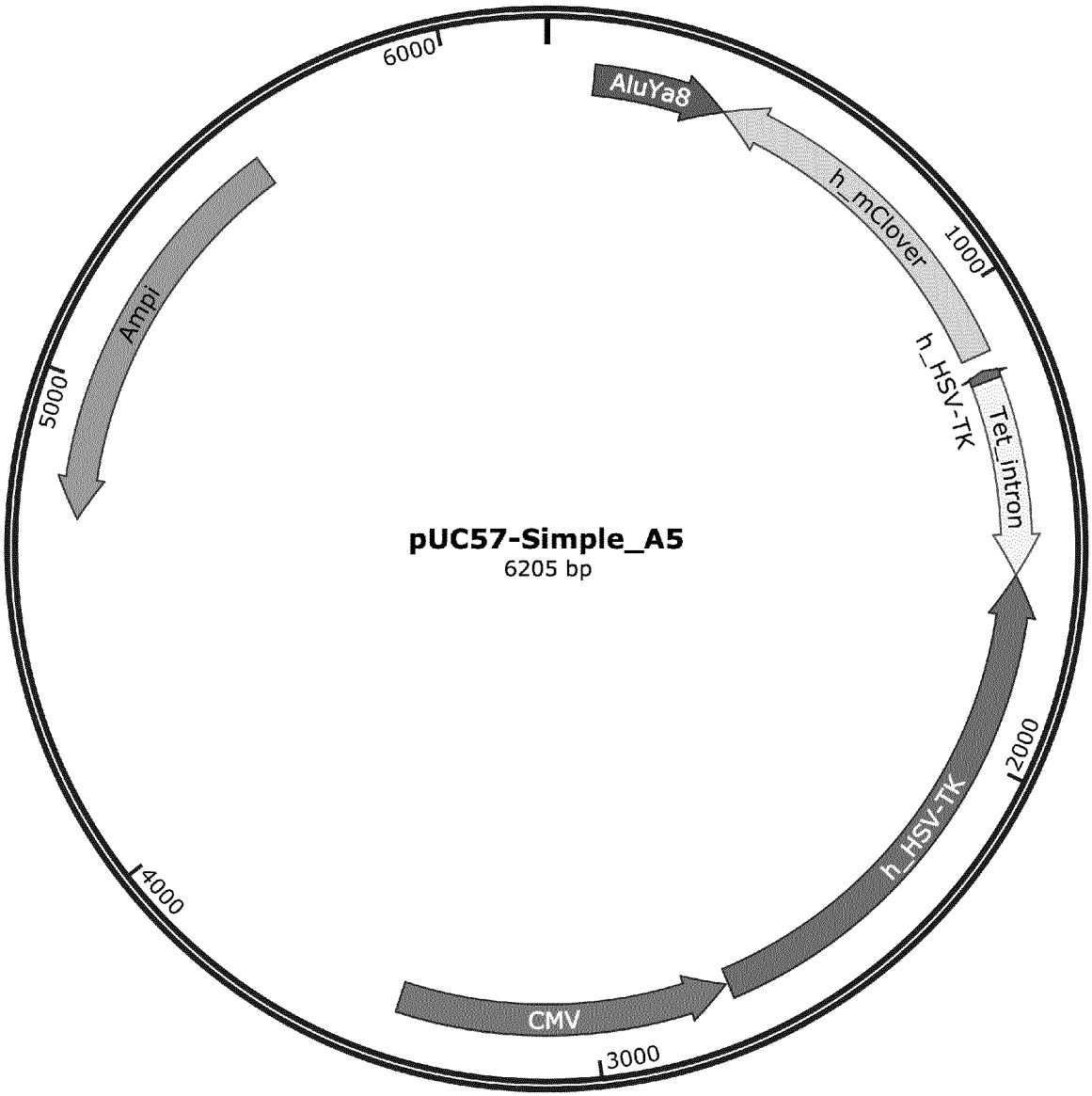

6000
AluYa8
h_mClover
1000
h_HSV-TK
Tet_intron
Ampi
5000
pUC57-Simple_A5
6205 bp
2000
h_HSV-TK
4000
CMV
3000

Figure 1(A)

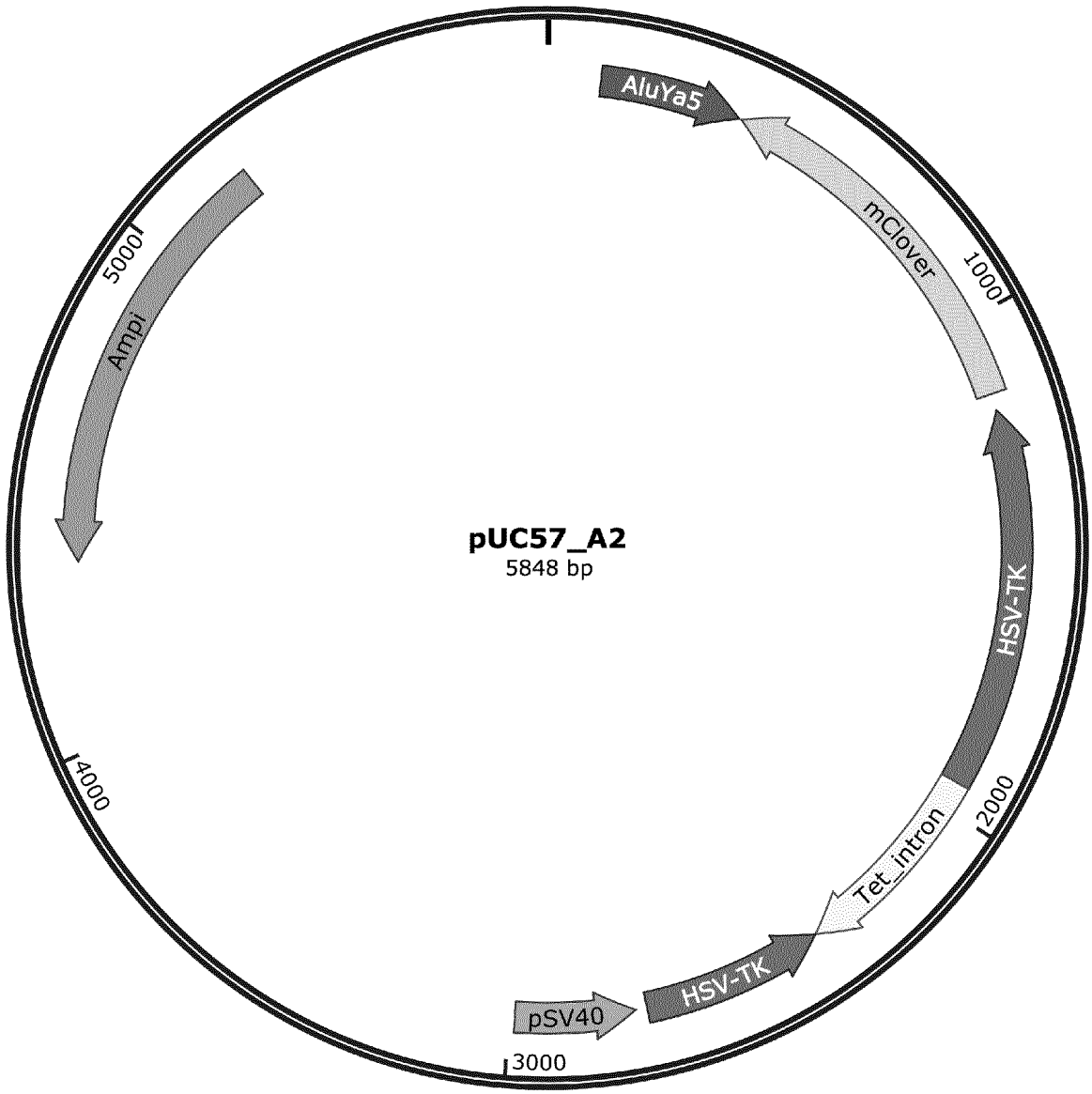

AluYa5
mClover
1000
HSV-TK
2000
Tet_intron
HSV-TK
pSV40
3000
4000
Ampi
5000
pUC57_A2
5848 bp

Figure 1(B)

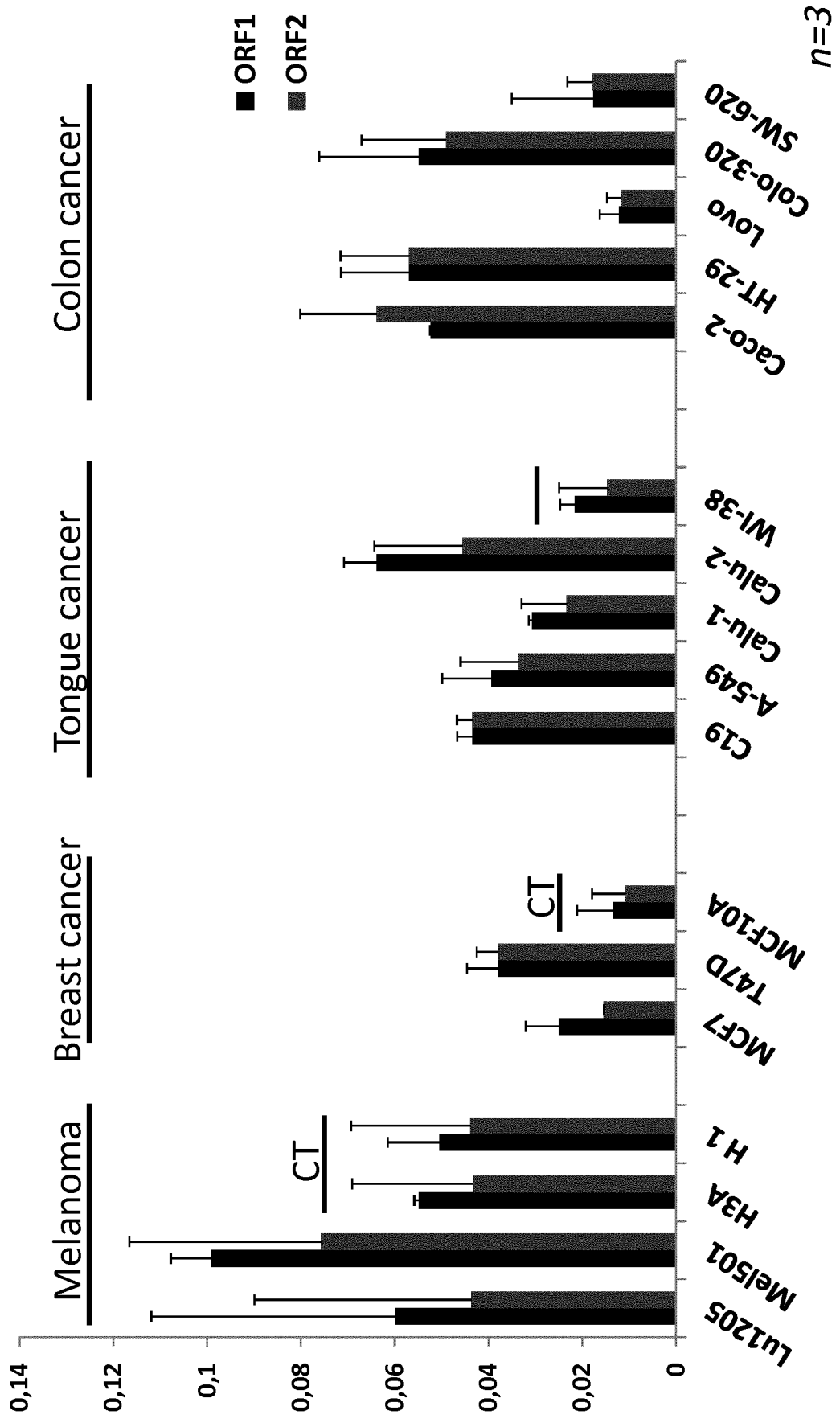

ORF1
ORF2
n=3
Melanoma
Breast cancer
Tongue cancer
Colon cancer
CT
CT
LU12OS
Mel501
H3A
H1
MCF7
T47D
MCF10A
C19
A-549
Calu-1
Calu-2
WI-38
Caco-2
HT-29
Lovo
Colo-320
SW-620
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0

Figure 2

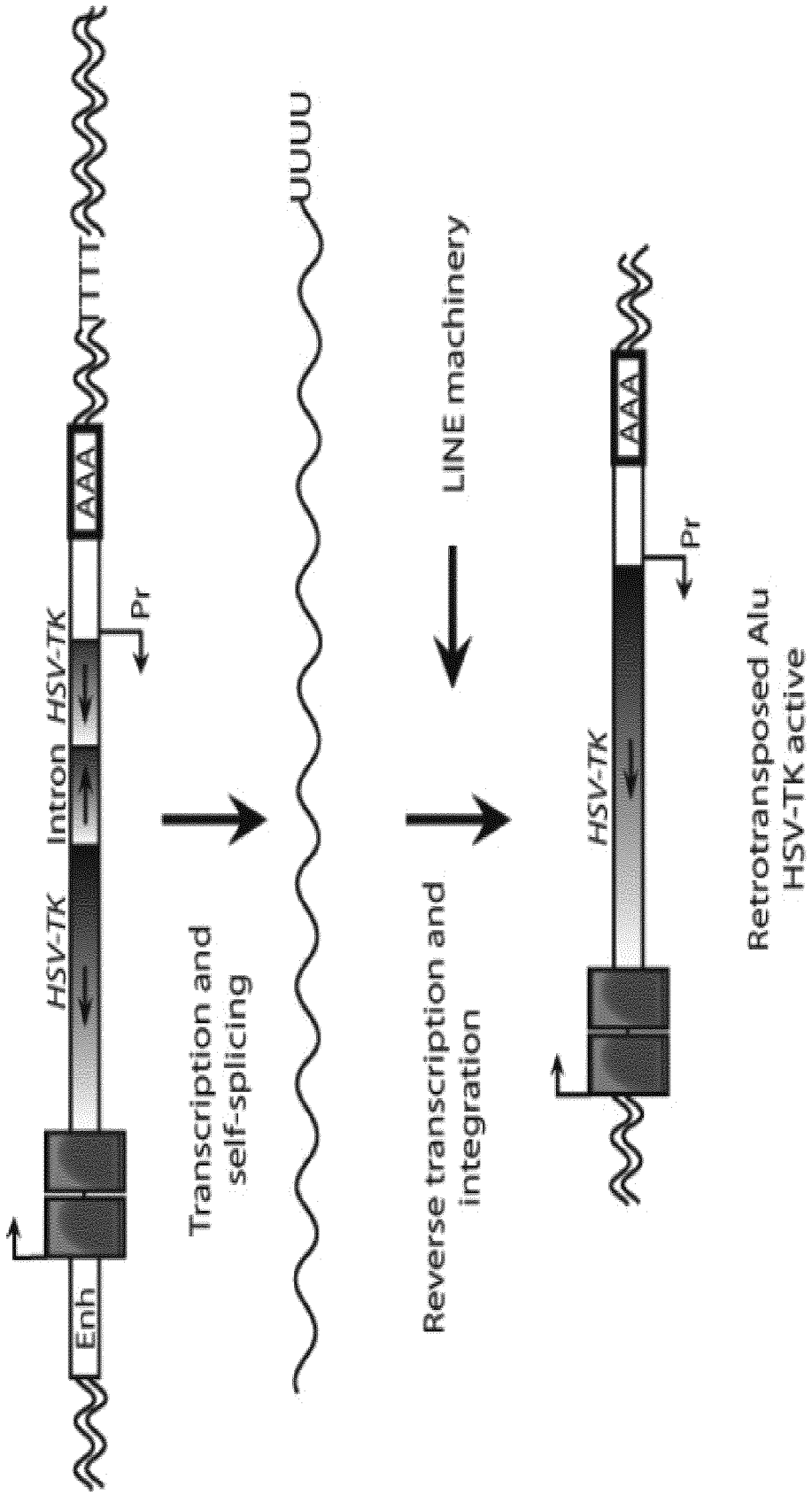

Enh
HSV-TK
Intron HSV-TK
Pr
AAA
TTTT
Transcription and self-splicing
UUUU
LINE machinery
Reverse transcription and integration
HSV-TK
Pr
AAA
Retrotransposed Alu HSV-TK active

Figure 3(A)

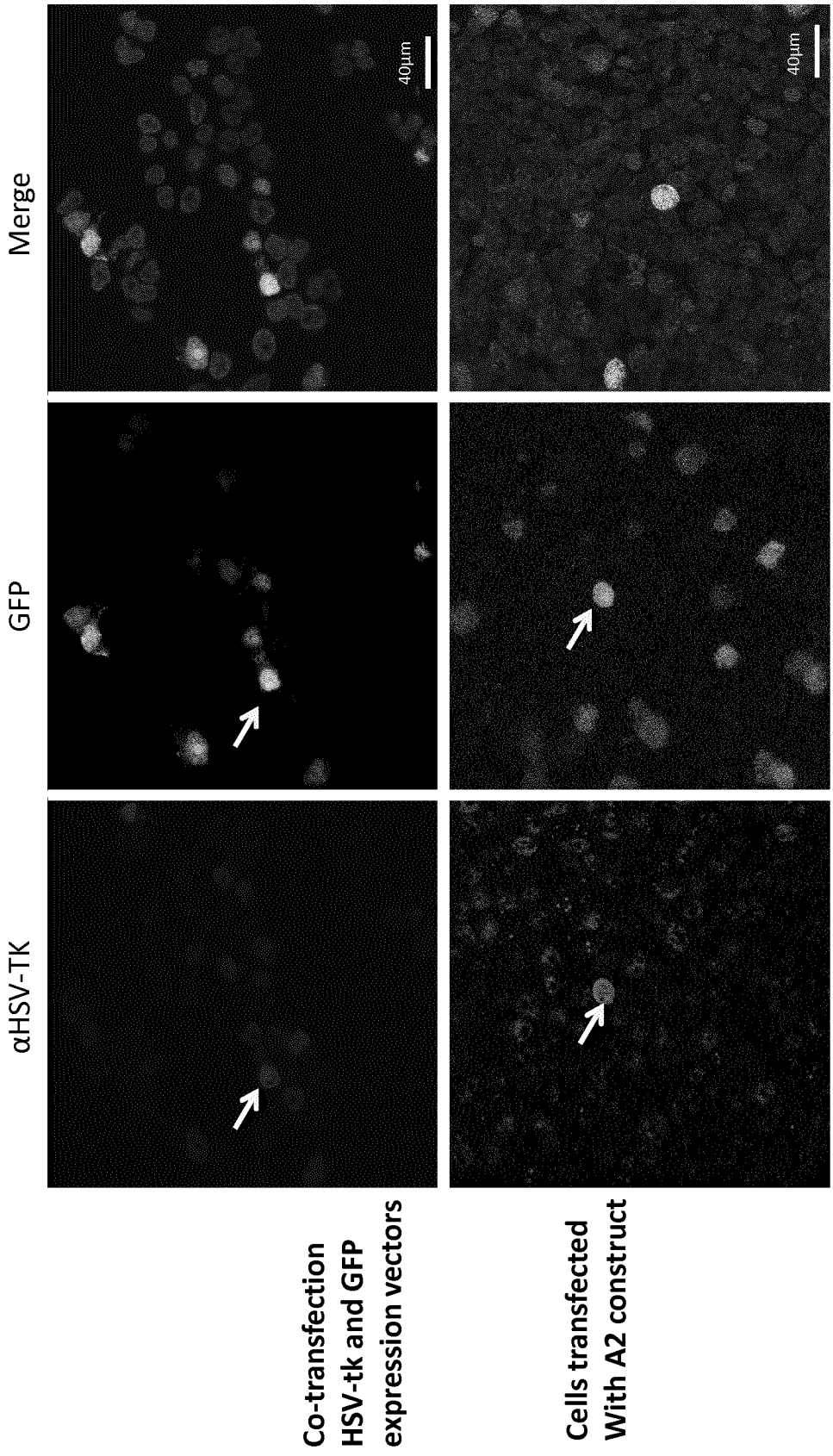

Merge
GFP
αHSV-TK
40µm
40µm
Co-transfection
HSV-tk and GFP
expression vectors
Cells transfected
With A2 construct

Figure 3(B)

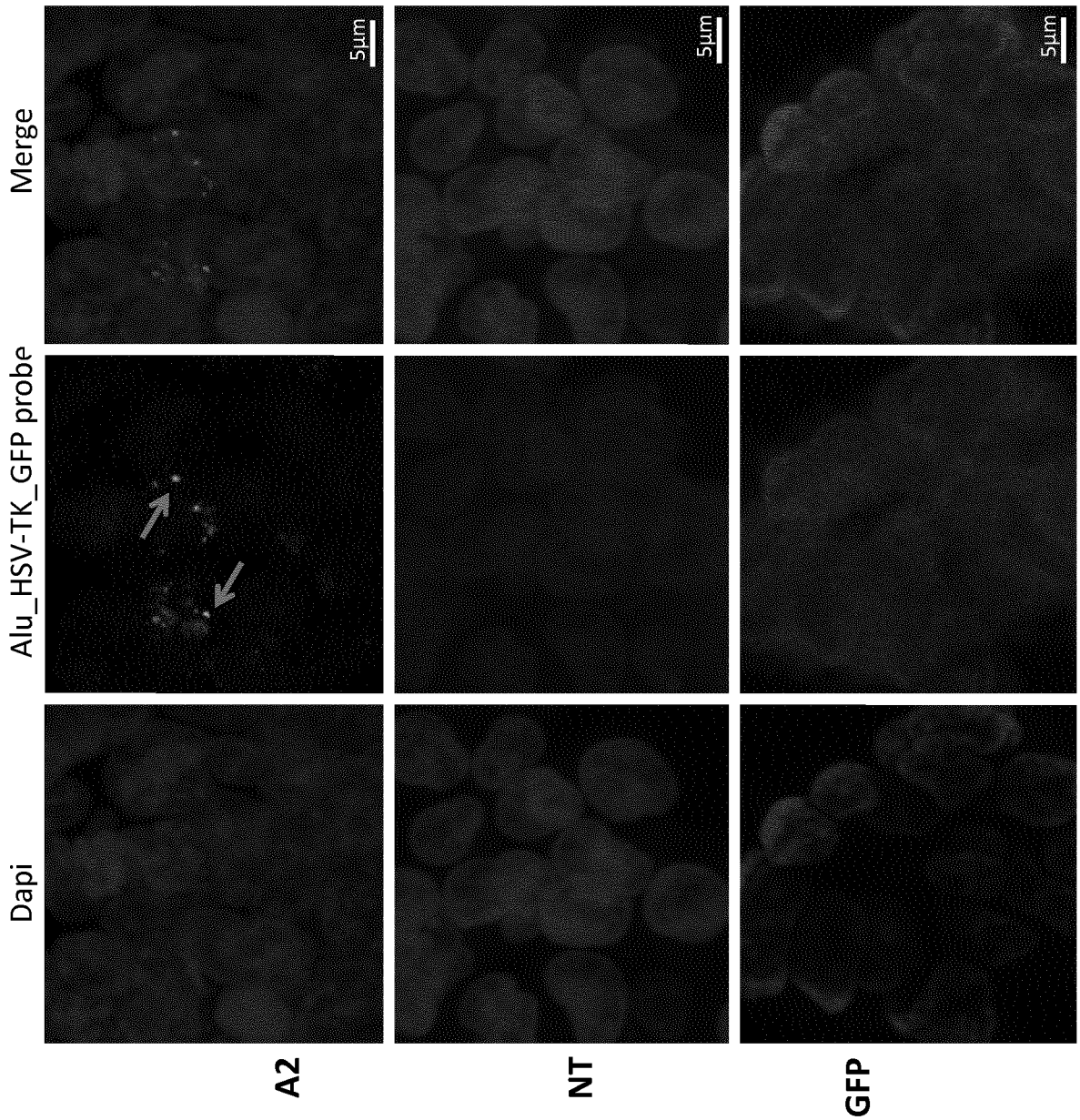


Figure 4

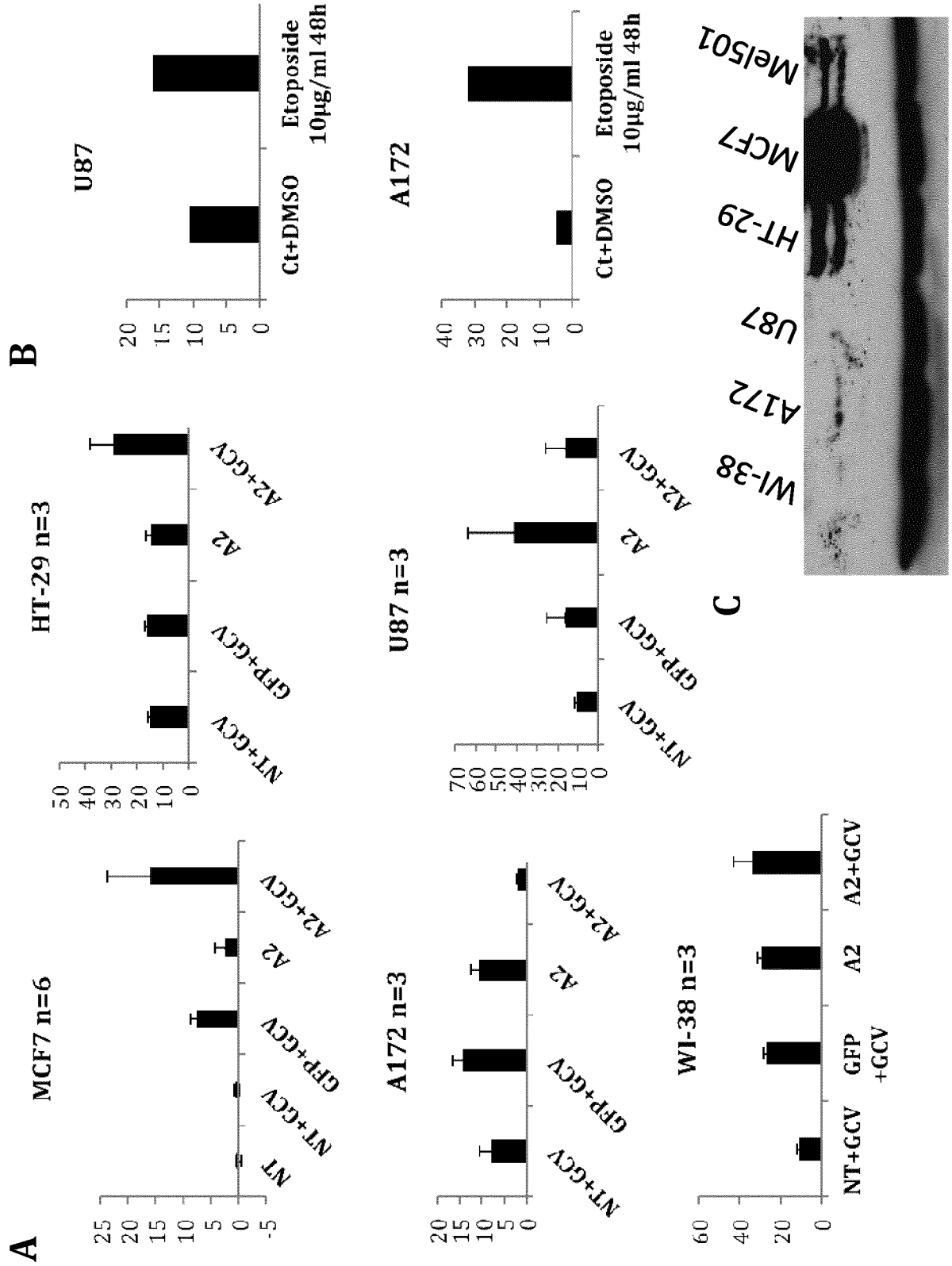

A
MCF7 n=6
NT
NT+GCV
GFP+GCV
A2
A2+GCV
A172 n=3
NT+GCV
GFP+GCV
A2
A2+GCV
WI-38 n=3
NT+GCV
GFP +GCV
A2
A2+GCV
B
HT-29 n=3
NT+GCV
GFP+GCV
A2
A2+GCV
U87 n=3
NT+GCV
GFP+GCV
A2
A2+GCV
U87
Ct+DMSO
Etoposide 10µg/ml 48h
A172
Ct+DMSO
Etoposide 10µg/ml 48h
C
WI-38
A172
U87
HT-29
MCF7
Mel501

Figure 5

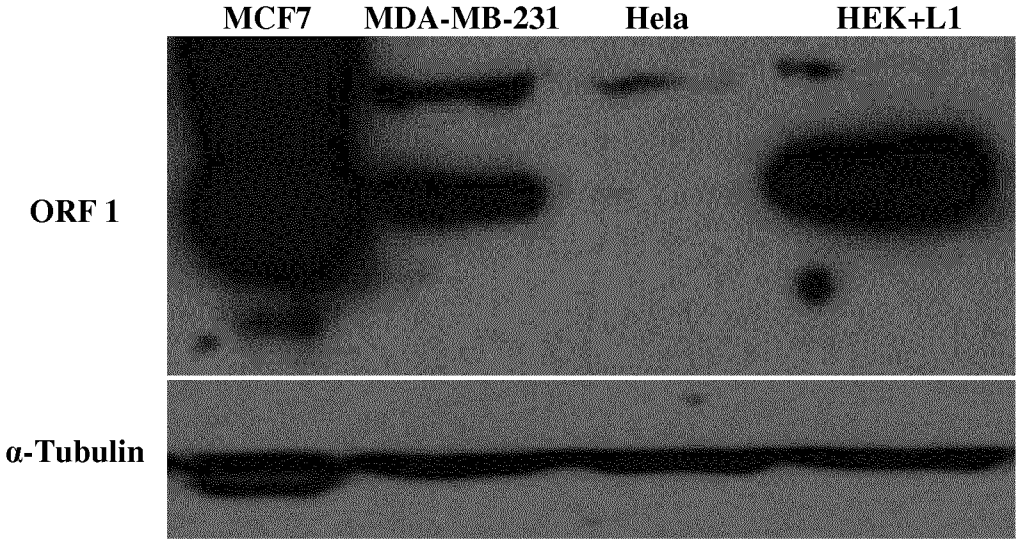
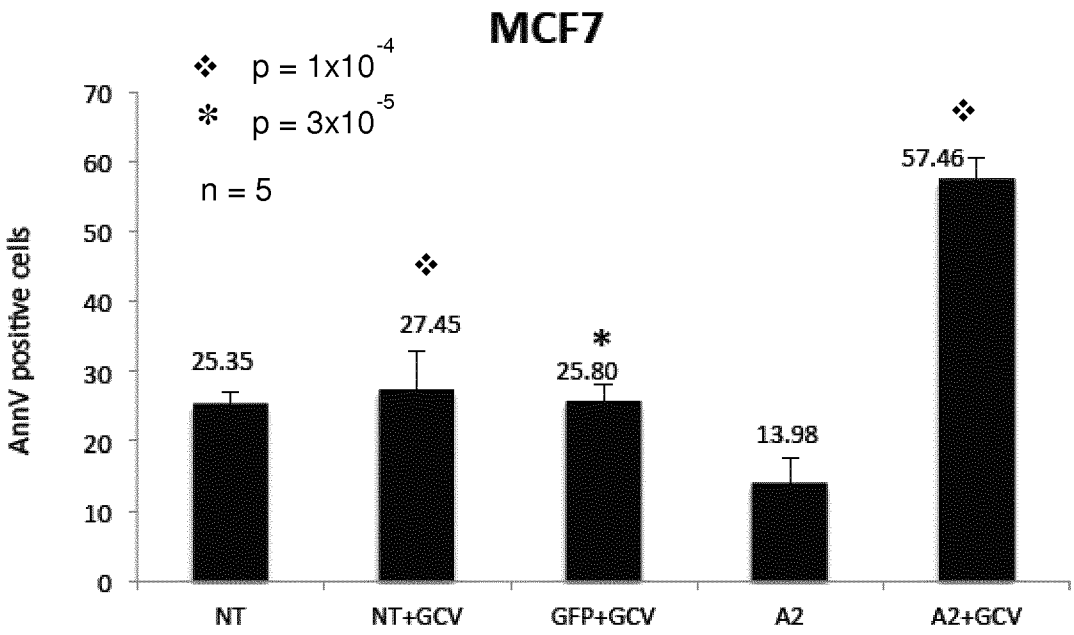


Figure 6

(A)


MCF7
❖ p = 1x10-4
* p = 3x10-5
n = 5
AnnV positive cells
70
60
50
40
30
20
10
0
25.35
27.45
25.80
13.98
57.46
NT
NT+GCV
GFP+GCV
A2
A2+GCV

(B)

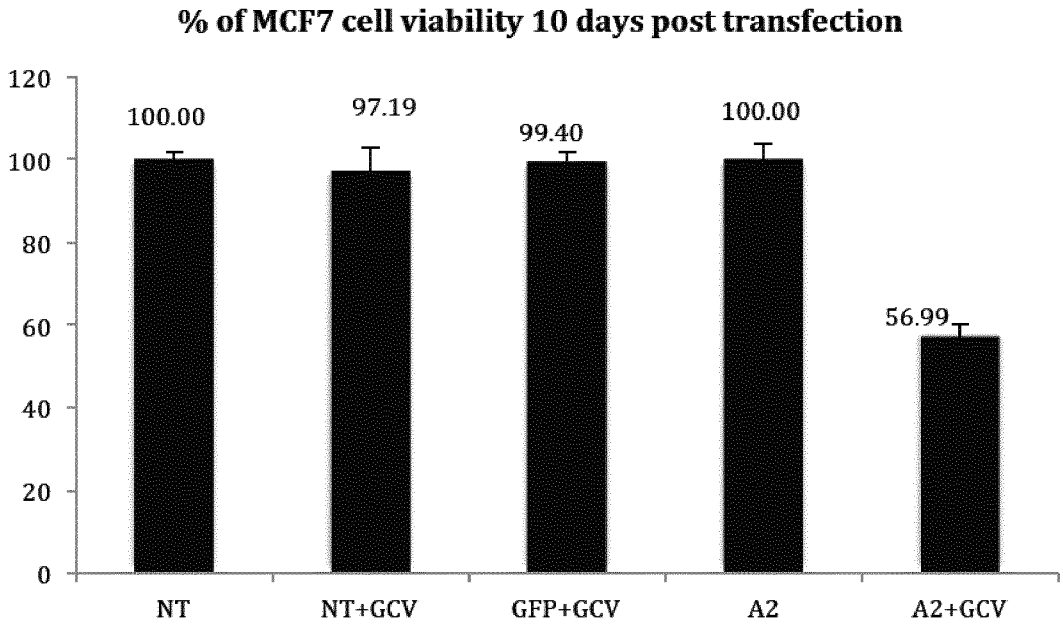

% of MCF7 cell viability 10 days post transfection
120
100
80
60
40
20
0
100.00
97.19
99.40
100.00
56.99
NT
NT+GCV
GFP+GCV
A2
A2+GCV

Figure 7

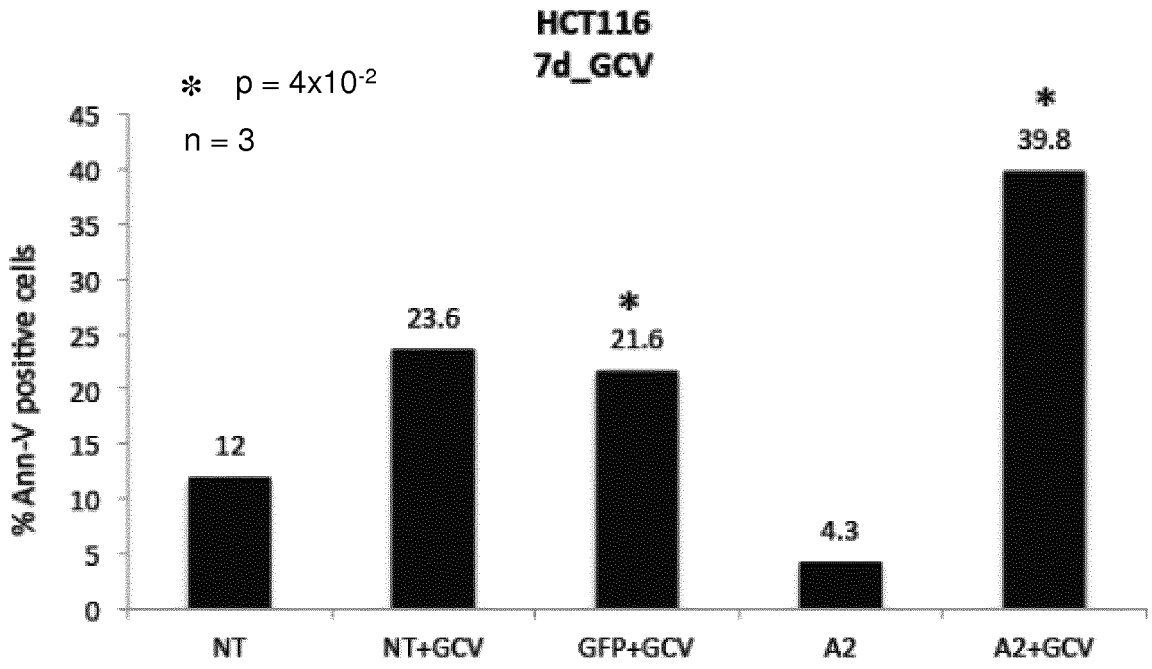

HCT116
7d_GCV
* p = 4x10-2
n = 3
% Ann-V positive cells
45
40
35
30
25
20
15
10
5
0
12
23.6
*
21.6
4.3
*
39.8
NT
NT+GCV
GFP+GCV
A2
A2+GCV

Figure 8

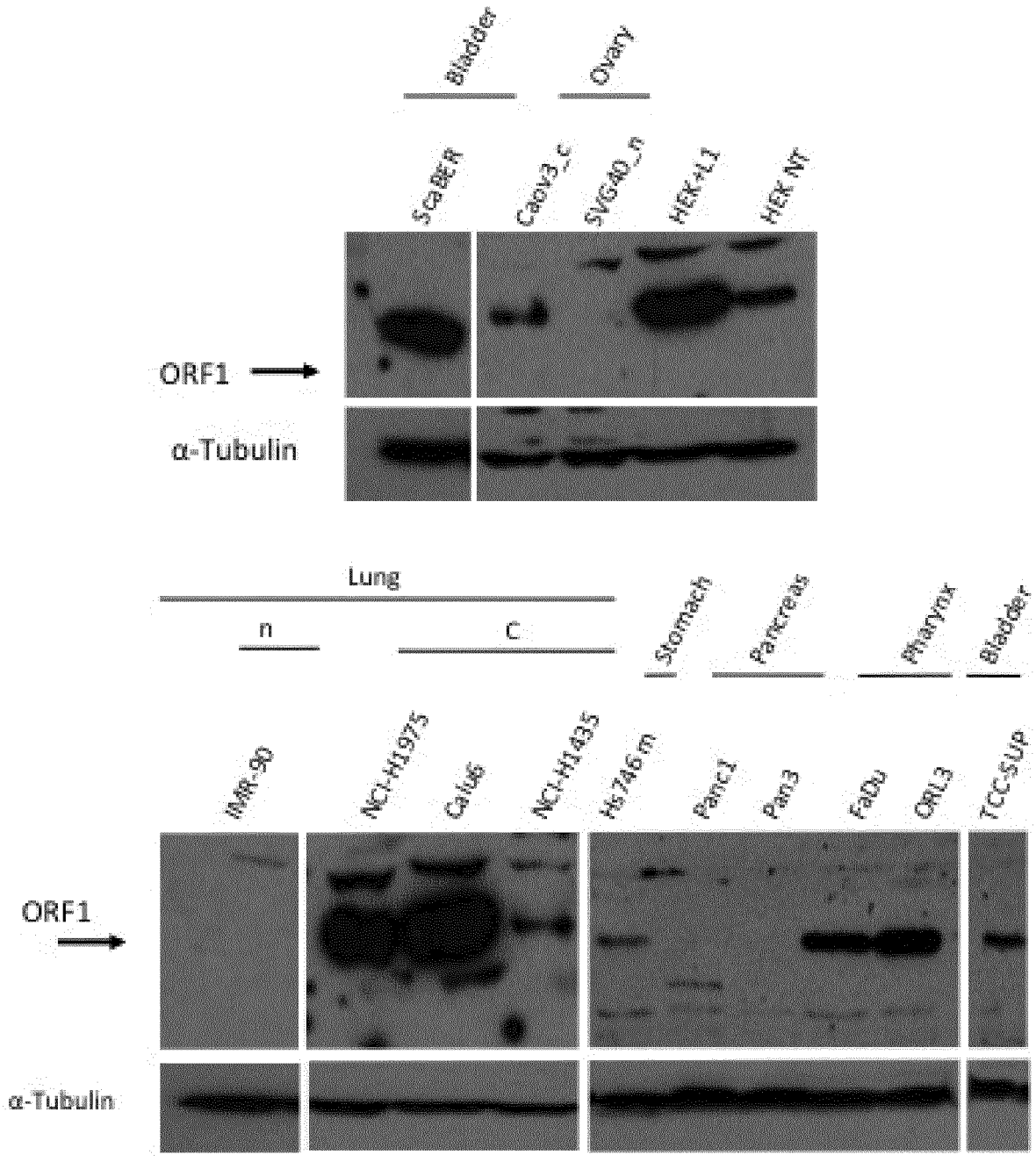

Bladder
Ovary
ScaBER
Caov3_c
SVG40_n
HEK+L1
HER NT
ORF1
α-Tubulin
Lung
n
C
Stomach
Pancreas
Pharynx
Bladder
IMR-90
NCI-H1975
Calu6
NCI-H1435
Hs746 m
Panc1
Pan3
FaDu
ORL3
TCC-SUP
ORF1
α-Tubulin

Figure 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/069734

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/85 A61P35/00 A61K48/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VINCENT A STREVA ET AL: "LINE-1 and Alu retrotransposition exhibit clonal variation", MOBILE DNA, BIOMED CENTRAL LTD, LONDON, UK, vol. 4, no. 1, 3 June 2013 (2013-06-03), page 16, XP021157319, ISSN: 1759-8753, DOI: 10.1186/1759-8753-4-16 figures 1B, 1D ----- -/--	13-15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 October 2015	30/10/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brouns, Gaby

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/069734

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARIE DEWANNIEUX ET AL: "LINE-mediated retrotransposition of marked Alu sequences", NATURE GENETICS, vol. 35, no. 1, 3 August 2003 (2003-08-03) , pages 41-48, XP055168712, ISSN: 1061-4036, DOI: 10.1038/ng1223 cited in the application	13-15
A	figures 1, 2	1-12, 16-19
A	RODIC NEMANJA ET AL: "Long Interspersed Element-1 Protein Expression Is a Hallmark of Many Human Cancers", AMERICAN JOURNAL OF PATHOLOGY, vol. 184, no. 5, May 2014 (2014-05), pages 1280-1286, XP055222292, page 1285, left-hand column, paragraph 1 figure 2	1-19
A	WO 2009/056321 A1 (BUNDESREPUBLIK DEUTSCHLAND LET [DE]; SCHUMANN GERALD [DE]; LAYER LILIA) 7 May 2009 (2009-05-07) examples 1-3 figures 2A, 2B claim 24	1-19
A	US 2003/017597 A1 (KASAHARA NORIYUKI [US] ET AL) 23 January 2003 (2003-01-23) figure 1	11
A	KARLA JOHANNING ET AL: "Potential for Retroposition by Old Alu Subfamilies", JOURNAL OF MOLECULAR EVOLUTION, vol. 56, no. 6, 1 June 2003 (2003-06-01), pages 658-664, XP055168683, ISSN: 0022-2844, DOI: 10.1007/s00239-002-2433-y page 659, left-hand column	1-4,7-19
A	KANG Y ET AL: "The piggyBac transposon is an integrating non-viral gene transfer vector that enhances the efficiency of GDEPT", CELL BIOLOGY INTERNATIONAL, ACADEMIC PRESS, GB, vol. 33, no. 4, 1 April 2009 (2009-04-01), pages 509-515, XP026062789, ISSN: 1065-6995, DOI: 10.1016/J.CELLBI.2009.01.017 [retrieved on 2009-02-12] cited in the application	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/069734

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009056321 A1	07-05-2009	AT 549410 T	15-03-2012
		EP 2055784 A1	06-05-2009
		EP 2205750 A1	14-07-2010
		WO 2009056321 A1	07-05-2009
US 2003017597 A1	23-01-2003	US 6576463 B1	10-06-2003
		US 2003017597 A1	23-01-2003