

CANCER THERAPEUTIC TARGETING USING MUTANT P53-SPECIFIC SIRNAS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore provisional application No. 10201801432S, filed on 21 February 2018, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of molecular biology. In particular, the present invention relates to the use of biomarkers for the detection and diagnosis, and siRNAs for the treatment of cancer.

BACKGROUND OF THE INVENTION

[0003] A large number of genomic alterations have been identified across almost all cancer types, through the pan-cancer genome sequencing efforts. This has led to the identification and association of many of these mutations as potential drivers that are casually involved cancer development. Some of the identified alterations in oncogenes have been subjected to therapeutic targeting through the development of inhibitory molecules or blocking antibodies, which have had huge initial success in the treatment of cancers bearing these mutations, forming the basis of precision medicine in oncology. However, one challenge of such an approach of using inhibitors or blocking antibodies for therapeutic targeting is that they are not entirely specific for the mutant form of the protein, but rather, are much effective in mutant protein expressing cells due to the elevated activity or expression of the mutant protein over its wild-type (WT) counterpart. As a result, this has the potential to lead to undesirable side effects on the multiple cell types that express the WT protein.

[0004] An ideal drug against a mutated protein would therefore be one that will only affect the functioning of the mutant form, without any effects on the WT version. However, up till now, there are no drugs or molecules that have been generated that are capable of such high specificity. Nonetheless, no routine technology to generate “mutant-only”-specific reagents has been available to date.

[0005] The era of precision medicine has promoted the development of many drugs that are specific for the highly active mutant forms of proteins. While spectacular results have initially been achieved, two major issues with specificity remain. Firstly, the drugs generated

against a particular protein (often a kinase) have almost always an impact on other cellular targets. Moreover, many of these drugs, though very effective on the mutant and active forms of the proteins, also have significant impact on the wild-type counterparts, as has been shown for c-Kit. Hence, though effective, the impact of these inhibitors on the wild-type form or other closely related targets will unabatedly lead to unwanted side effects, reducing the promise of these reagents.

[0006] Hence, there is a need for reagents that are highly specific for the mutated versions of the protein, with little or no cross-reactivity to the wild-type form for use in treating hyperproliferative disease.

SUMMARY OF THE INVENTION

[0007] In one aspect, the present invention refers a nucleic acid sequence for targeting a single point mutation within a target gene, wherein the target gene is one or more tumour suppressor genes; wherein the tumour suppressor gene is p53, and wherein the site of the point mutation is selected from the group consisting of R249 (p53), R248 (p53), R273 (p53) and R175 (p53).

[0008] In another aspect, the present invention refers to a method of treating cancer in a subject, the method comprising administering to the subject one or more nucleic acid sequences as disclosed herein, wherein the nucleic acid sequences target one or more point mutations sites within a target gene, wherein the target gene is a tumour suppressor gene.

[0009] In yet another aspect, the present invention refers to a method of identifying a subject susceptible to treatment, wherein the method comprises i) identifying one or more single point mutations within a target gene, wherein the target gene is one or more tumour suppressor genes; wherein the tumour suppressor gene is p53, and wherein the site of the one or more point mutations is selected from the group consisting of R249 (p53), R248 (p53), R273 (p53) and R175 (p53); ii) administering to the subject one or more nucleic acid sequences as disclosed herein, wherein the nucleic acid sequences target one or more point mutations sites within the target gene, wherein the presence of the one or more point mutations in the target gene indicate that the subject is susceptible to treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0011] **Fig. 1** provides data showing siRNA sequences selected by siRNA walk to specifically target various p53 hot-spot mutants. (A) shows the nucleotide sequence of wild-type (WT) and the respective p53 mutants (i.e. R175H; R248W; R249S and R273H) are indicated in each case, followed by the p53 allele-specific siRNA sequences shortlisted to target each mutant. Both the WT and the mutated nucleotide residue are highlighted in bold. The pan-p53 siRNA (si-p53) and the scrambled (scr) siRNA (si-scr) sequences are indicated at the bottom. (B) shows images of immunoblots performed for each siRNA shown in (A). Each siRNA was transfected into isogenic H1299 cell lines stably expressing the indicated p53 mutants. The cell lysate was then analysed for p53 expression by immunoblotting, 72 hours post-transfection, using anti-p53 antibody (DO-1). Temperature sensitive (TS) WT p53 expressing cells were used as a WT control. The mutant-specific siRNAs that showed specific and improved knock-down activity are indicated with an asterisk (“*”). One representative blot of at least three independent experiments is shown. Actin is shown as loading control, and (-) represent cells only without any siRNA transfection. For each sample, the ratio of p53 to Actin band intensity was calculated and normalized to the ratio of si-scr control. Values represent normalized fold change.

[0012] **Fig. 2** depicts immunoblot data showing the silencing efficacy of mutant-specific siRNAs on endogenous mutant p53. Panels (A) to (D) show immunoblot results of siRNAs against R175H, R248W, R249S and R273H, respectively. Mutant siRNAs were transfected in the three cell lines with WT p53 expression, and in three cell lines expressing the indicated p53 mutants. Silencing efficacy was evaluated by immunoblotting as described above. One representative blot of at least three independent experiments is shown. Mutant p53 status of cell lines is highlighted below the blots and described in Table 1.

[0013] **Fig. 3** presents flow cytometry graphs showing that allele-specific silencing of mutant p53 expression leads to cell death. Flow cytometric analysis of the sub-G1 DNA content (indicative of apoptosis) in cells were quantified 72 hours post-transfection of the indicated siRNAs in the indicated cell lines. Representative histograms are shown from one experiment out of at least three independent repeats. % sub-G1 cells are indicated in the histogram (M1).

[0014] **Fig. 4** provides histograms illustrating that mutant p53-specific silencing leads to activation of p53 canonical target genes in mutant p53 expressing cells. (A) shows HCT116 cells expressing WT p53 were transiently transfected with siRNAs targeting the four hot-spot p53 mutants or the control scrambled siRNA or p53-specific siRNA. Cells were collected 72 hours later for mRNA analysis of the indicated target genes by quantitative real time PCR. (B) shows AU565, 786-O, BT549 and ASPC1 cell lines expressing the indicated p53 mutants were similarly transfected and analysed. Relative expression of the target genes is shown. All experiments were normalized to GAPDH and carried out in triplicates. Bar diagrams show the mean $\pm$ standard deviation of three independent experiments. * indicates p value of <0.05 , ** <0.005 ; and *** <0.001 , with $n=3$ samples per group.

[0015] **Fig. 5** shows data depicting the growth suppressive effect of mutant p53-specific shRNAs. (A) shows immunoblots of the indicated cell lines, which were transfected with shRNA expressing pan-p53 (sh-p53) shRNA, scrambled control, the respective mutation-specific shRNAs or empty vector (-). The cells were harvested 48 hours later and analysed for efficiency of silencing by immunoblotting. (B) shows images of parallel cultures of cellular colonies which were stained with crystal violet solution 5 days post shRNA transfection and visualized. Representative images are shown from one experiment, out of at least three independent experiments (b), and quantified.

[0016] **Fig. 6** depicts data showing the relief of dominant-negative effects of mutant p53 by mutant p53-specific silencing. (A) shows immunoblots of RKO^{+/-} and RKO^{+248W} cells which were transfected with control, pan-p53 (sh-p53) or R248W-specific shRNAs (sh-4), and analysed as described above for efficacy of silencing. Data on colony growth is shown in panel (B), and results of p53 target gene expression analysis are shown in panel (C). Cell death was analysed without (panel D) or with cisplatin (CDDP) treatment (panel E). Percentage (%) of sub-G1 cells are indicated on the histograms (as represented by M1). Representative data are shown from three independent experiments. Bar diagrams show the mean $\pm$ standard deviation of the three independent experiments. ** indicates p value of <0.005 ; and *** <0.001 , with $n = 3$ samples per group.

[0017] **Fig. 7** presents data showing that mutant p53-specific silencing retards tumour growth *in vivo*. Mutant p53-specific silencing retards tumour growth *in vivo*. (A) & (B) RD, PLC-PRF5, and H1975 cell lines were transduced with scrambled or the indicated mutant-p53-specific shRNAs and were collected 3 days later, and cells [RD (4×10^6), PLC-PRF5 (3×10^6) and H1975 (5×10^6)] as a mixture of 75 μ l cells in PBS and 75 μ l Matrigel were injected into the flanks of SCID mice, and tumour growth was monitored regularly. Sizes of

tumours are indicated in the graphs (A). Tumours harvested at end point in each case were used for H&E or anti-p53 staining on RD tumours (B). Values represent mean + SD. n = 4 (per group for RD and H1975 cells) and n = 5 (for PLC cells). *** indicates p value of <0.001.

[0018] **Fig. 8** shows immunoblot data on the silencing efficacy of mutant-specific siRNAs on endogenous mutant p53 expression. siRNAs against R175H (si-1 & 2), R248W (si-3 & 4), R249S (si-5 & 6) and R273H (si-7 & 8), were transfected in the indicated cell lines expressing the indicated p53 mutants, and the silencing efficacy was evaluated by immunoblotting as described. One representative blot of at least two independent experiments is shown. Mutation p53 status of cell lines is highlighted below the blots and described in Table 1. For each sample, the ratio of p53 to Actin band intensity was calculated and normalized to the ratio of si-scr control. Values represent normalized fold change

[0019] **Fig. 9** shows further flow cytometric results of the evaluation of effects of mutant-specific siRNAs on cell death in cell lines expressing various mutant p53. Flow cytometric analysis of the sub-G1 DNA content (indicative of apoptosis) in cells were quantified 72 hours post-transfection of the indicated siRNAs (as described herein) in the indicated cell lines. Representative histograms are shown. % sub-G1 cells are indicted in the histogram (M1 for the HCC1395 cells and M2 for all the other cell lines).

[0020] **Fig. 10** shows further flow cytometric data pertaining to cisplatin treatment potentiates cell death upon mutant p53 silencing. Flow cytometric analysis of the sub-G1 DNA content (indicative of apoptosis) was performed in HCT116, AU565, 786-O, BT549 and H1975 cells. These cells were transfected with the indicated siRNAs and treated with cisplatin 48 hours post-transfection, for another 24 hours. Representative histograms are shown from one experiment out of at least three independent repeats. Percentage (%) of sub-G1 cells are indicted in the histogram (M1).

[0021] **Fig. 11** shows histograms showing real-time PCR data on the depletion of mutant allele expression leads to activation of p53 transcriptional targets. qRT-PCR for p53 target genes such as p21, Mdm2 and Noxa was performed on the indicated cells lines that were transfected with the various siRNA, and subsequently treated without or with cisplatin (CDDP), as described herein. All experiments were normalized to GAPDH and carried out in triplicates, and relative expression of the target genes is shown. Bar diagrams show the mean $\pm$ standard deviation of three independent experiments. * indicates *p* value of <0.05; **<0.005; and ***<0.001, with n=3 samples per group.

[0022] **Fig. 12** shows data indicating that allele-specific mutant p53-specific shRNAs induces cell death and are effective on various mutant nucleotides at the same residues. (A) shows histograms with the results of flow cytometric analysis of the sub-G1 DNA content (indicative of apoptosis) in the indicated cell lines, which were transfected with the indicated shRNAs was performed 72 hours post-transfection. Representative histograms show the mean $\pm$ standard deviation of three independent experiments. (B) to (D) show data from HEC1A cells expressing the R248Q mutant p53, which were transfected with the indicated shRNAs and analysed for mutant p53 expression (B), colony growth (C) and apoptosis in the absence or presence of CDDP treatment (D). Representative results from one of three independent experiments are shown. Bar diagrams show the mean $\pm$ standard deviation of three independent experiments. sh-4 is the R248 specific siRNA

[0023] **Fig. 13** shows data illustrating the relief of the dominant-negative effects of mutant p53 by mutant p53-specific silencing. HCT116+/- and HCT116+/R248W cells were transfected with control, pan-p53 (sh-p53) or R248W-specific shRNAs (sh-4), and analysed as described for efficacy of silencing (A), colony growth (B), and p53 target gene expression (C). Cell death was analysed without (panel D) or with cisplatin (CDDP) treatment (panel E). Percentage (%) of sub-G1 cells are indicated on the histograms (as represented by M1). Representative data are shown from three independent experiments. Bar diagrams show the mean $\pm$ standard deviation of the three independent experiments. * indicates *p* value of <0.05; **<0.005; and ***<0.001, with n=3 samples per group.

[0024] **Fig. 14** shows data depicting the efficacy of siRNA-6 on the various R249 mutants. (A) shows a table showing the various possible mutations found at position R249 of p53 in human cancers, and the nucleotide sequences for each amino acid possibility. R249 can therefore be mutated to R249S, R249G and R249M. (B) shows various histograms showing the frequency of the various R249 mutations in human cancers, as mutation counts. (C) shows results of immunoblotting analysis. si-6 and the control sip53 and si-scr siRNAs were transfected into H1299 cell lines that were co-transfected 24 hours later with the various R249 mutant cDNA constructs or the WT p53 construct. The cell lysate was then analysed for p53 expression by immunoblotting, 72 hours post-siRNA transfection, using anti-p53 antibody (DO-1). One representative blot of at least two independent experiments is shown. Actin is shown as loading control, and (-) represent cells only without any siRNA transfection.

[0025] **Fig. 15** shows data depicting the efficacy of siRNA-8 on the various R273 mutants. (A) is a table showing the various possible mutations found at position R273 of p53

in human cancers, and the nucleotide sequences for each amino acid possibility. R273 can therefore be mutated to R273H and R273L. (B) shows various histogram showing the frequency of the various R273 mutations in human cancers, as mutation counts. (C) shows results of immunoblotting analysis. si-8 and the control sip53 and si-scr siRNAs were transfected into H1299 cell lines that were co-transfected 24 hours later with the various R273 mutant cDNA constructs or the WT p53 construct. The cell lysate was then analysed for p53 expression by immunoblotting, 72 hours post-siRNA transfection, using anti-p53 antibody (DO-1). One representative blot of at least two independent experiments is shown. Actin is shown as loading control, and (-) represent cells only without any siRNA transfection.

[0026] **Fig. 16** provides histograms illustrating that mutant p53-specific silencing leads to activation of p53 canonical target genes in mutant p53 expressing cells. (A) shows histograms of H1299 cells transfected with the R273L and R273H p53 mutant cDNAs 24 hours after transfection of the si-8 and the control scrambled siRNA or p53-specific siRNA control siRNAs. Cells were collected 72 hours post-siRNA transfection for mRNA analysis of the indicated target genes by quantitative real time PCR. (B) similarly shows histograms of H1299 cells transfected with the R249G, R249M or R249S p53 mutant cDNAs along with the si-6 and the control scrambled siRNA or p53-specific siRNA control siRNAs. Cells were collected 72 hours post-siRNA transfection for mRNA analysis of the indicated target genes by quantitative real time PCR. (C) shows histograms of H1299 cells transfected with WT p53 cDNA along with si-2, si-4, si-6, si-8 and the control scrambled siRNA or p53-specific siRNAs. Cells were collected 72 hours post-siRNA transfection for mRNA analysis of the indicated target genes by quantitative real time PCR. Relative expression of the target genes is shown. All experiments were normalized to GAPDH and carried out in triplicates.

[0027] **Fig. 17** shows a table summarising the efficacy of mutant p53-specific siRNAs on mutations within the same amino acid. Y: represents yes (in other words, siRNA is effective in targeting the listed mutant).

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0028] Mutations in *Tp53* compromise therapeutic response, due either to the dominant-negative effect over the functional wild-type allele; or as a result of the survival advantage conferred by mutant p53 to which cancer cells become addicted. Thus, targeting mutant p53 represents an effective therapeutic targeting of over half of all cancers. A series of small-

interfering-RNAs, capable of targeting p53 hot-spot mutants, have been generated. These mutant-p53-sepcific siRNAs (MupSi) are highly specific in silencing the expression of the intended mutants without affecting wild-type p53. Without being bound by theory, it is thought that functionally, these MupSis induce cell death by abrogating both the addiction to mutant p53 and the dominant-negative effect; and retard tumour growth in xenografts when administered in a therapeutic setting.

[0029] Thus, in one example, there is disclosed a nucleic acid sequence for targeting a single point mutation within a target gene. In another example, the target gene is one or more tumour suppressor genes. In yet another example, the tumour suppressor gene is p53.

[0030] Functionally, and without being bound by theory, these mutant-p53-sepcific siRNAs induce cell death by abrogating both the addiction to mutant p53 and the dominant-negative effect; and retard tumour growth in xenografts when administered in a therapeutic setting, demonstrating that mutation-specific siRNAs can be generated and effectively used to improve therapeutic response, a strategy that could be widely applicable.

[0031] In one example, the nucleic acid sequence results in any one or more of the following effects, which are, but are not limited to, cell death, abrogation of addiction, activation of any one or more of the target genes, relief of a dominant negative effect, increased sensitivity to one or more anti-cancer agents, and retardation or halting of tumour growth. In another example, the nucleic acid sequence is capable of substantially silencing mutant tumour suppressor gene alleles. In another example, the nucleic acid sequence as disclosed herein silences mutant suppressor gene alleles. In yet another example, the nucleic acid sequence as disclosed herein silences mutant suppressor gene alleles, without affecting the corresponding wild-type allele.

[0032] As used herein, the term “mutation” or “mutated” or “genetic alteration” refers to a natural or artificial modification, or genetic alteration of the genome or part of a nucleic acid sequence of any biological organism, virus or extra-chromosomal genetic element. This mutation can be induced artificially using, but not limited to, chemicals and radiation, but can also occur spontaneously during nucleic acid replication in cell division. Mutations may or may not produce discernible changes in the observable characteristics (phenotype) of an organism. There are various types of mutations known, which can either be small-scale mutations or large-scale mutations. Examples of small-scale mutations are, but are not limited to, substitution mutations, silent mutations, missense mutations, nonsense mutations, insertions, and deletions. Examples of large-scale mutations are, but are not limited to, amplifications, deletions, chromosomal translocations, interstitial deletions, chromosomal

inversions and mutations that result in a loss of heterozygosity. Mutations can also be grouped by their effect on the function of the resulting product. These include, but are not limited to, loss-of-function (inactivating) mutations, gain-of-function (activating) mutations, dominant-negative (antimorphic) mutations, lethal mutations and back or reverse mutations. Point mutations, for example, also known as single base modification, are a type of mutation that causes a single nucleotide base substitution, insertion, or deletion of the genetic material, DNA or RNA. The term “frame-shift mutation” indicates the addition or deletion of a base pair.

[0033] As used herein, the term “hot spot mutation” refers to a region or site within a DNA sequence that shows a statistically high propensity to mutate. Such highly frequent mutations can be found in, for example, the p53 gene across all cancer types. As an example, there are six sites found within the p53 gene. These hot spot mutation sites include R175, R248, R249, and R273. In one example, the site of the point mutation is but is not limited to, R249 (p53), R248 (p53), R273 (p53) and R175 (p53).

[0034] Thus, in one example, the mutation is a point mutation. In another example, the point mutation is a substitution mutation. In yet another example, the mutation is a hot spot mutation.

[0035] In another example, the point mutation is, but is not limited to, R175H (p53), R248W (p53), R273H (p53), R249S (p53), and combinations thereof. In another example, the point mutation is, but is not limited to, R249S (p53), R249G (p53), R249M (p53), R248W (p53), R248Q (p53), R273H (p53), R273L (p53) and R175H (p53).

[0036] Among the mutated genes in cancers, mutations in the tumour suppressor gene Tp53 (hereinafter referred to as p53) occur with the highest frequency, cementing its position as the critical gate-keeper gene whose functions have to be abrogated for cancers to develop. Mutations in p53 can occur almost on all of its 393 residues, and these mutations impact tumourigenesis in multiple ways. Firstly, mutations in p53 in the germ-line lead to cancer predisposition, as exemplified in the Li-Fraumeni syndrome, and in many model organisms. In addition, mutations in p53 have been associated with poor response to therapy, due often to the dominant-negative (DN) effects of the mutant protein over the remaining wild-type protein, which could be ameliorated by reducing the expression of the mutant form. Finally, cancer cells are often addicted to the presence of mutant p53 for survival and metastasis, and abrogation of many of the acquired gain-of-functions (GOF) of mutant p53 can reduce addiction and metastasis, thereby inducing tumour cell death and tumour load *in vivo*. However, GOF in itself appears not to be a universal phenomenon among all p53 mutants.

[0037] From the therapeutic perspective, mutant p53 would therefore be expected to be the prime target to treat cancers. However, the lack of interest to develop reagents to target mutant p53 stems from the fact that p53 is considered to be an “undruggable” transcription factor. This belief has hampered progress in the development of p53 targeting agents. Moreover, it was recently shown that not all mutants are equal in form and function, and that targeting mutant p53 would require a plethora of molecules, as opposed to a single agent capable of selectively targeting the various p53 mutants. Furthermore, in order to be effective, all of these molecules should not affect the functioning of the wild-type form. Thus, current technologies used in drug development have not been applied nor have they been successful in targeting mutant p53.

[0038] Small-interfering RNAs (siRNA) have been developed for many targets to silence their expression successfully, and as shown herein, can be seen as an avenue to target the various mutant p53. However, siRNAs that are capable of recognizing a single nucleotide change have not been generated routinely, due mainly to the inability to achieve specificity to target a single nucleotide change, without affecting the wild-type counterparts of the intended targets. These technologies have not been utilized routinely to generate reagents for multiple genetic alterations on the same gene. Therefore, the possibility of generating siRNAs that are specific for six hot-spot mutations, for example of p53 has been explored. Data provided herein demonstrates the generation of such mutant p53-specific siRNAs (referred to as MupSi), and demonstrate their utility in selectively silencing the expression of the intended mutant p53 forms, without cross-reactivity against other mutants or against the wild-type protein. Furthermore, these siRNAs have been used to demonstrate the amelioration of the dominant negative (DN) activity of mutant p53 over the wild-type form, thereby sensitizing tumour cells to therapeutic treatment. Moreover, they also abrogate the addiction of cancer cells to mutant p53 for survival, leading to cell death of tumour cells expressing mutant p53. Finally, it is shown that siRNAs can be used as therapeutic agents, and are capable of retarding tumour growth *in vivo* without having any side effects or organ toxicity (data not shown). The generation of such mutant p53-specific siRNAs (referred to as MupSi) is shown herein. Furthermore, their ability in selectively silencing the expression of the intended mutant p53 forms is demonstrated, without cross-reactivity against other mutants or against the wild-type protein. Furthermore, these RNAs have shown to ameliorate of the dominant negative (DN) activity of mutant p53 over the wild-type form, thereby resulting in a sensitisation of mutant tumour cells to therapeutic treatment. Moreover, these RNAs are also shown to abrogate the addiction of cancer cells to mutant p53 for survival, leading to cell

death of tumour cells expressing mutant p53. Finally, it is shown that these RNAs can be used as therapeutic agents, and are capable of retarding tumour growth in vivo without resulting in any side effects or organ toxicity. Together, this data demonstrates that mutation-specific RNAs, for example siRNAs can be routinely generated and that these mutant specific siRNA are effective in treating cancer.

[0039] The term “RNAi” refers to RNA interference, a process in which RNA molecules inhibit gene function. This interference is based on the ability of double-stranded RNA to interfere with, or suppress, the expression of a gene with a corresponding base sequence. For example, two types of small ribonucleic acid (RNA) molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are important to RNA interference. RNA molecules (or RNAs) are the direct products of genes, and these small RNAs can bind, for example, to other specific messenger RNA (mRNA) molecules, thereby either increase or decrease their activity, for example by preventing an mRNA from producing a protein.

[0040] As used herein, the term “RNA”, that is “ribonucleic acid” refers to an organic molecule consisting of along chain of nucleotides in which the sugar is ribose (or variations thereof) and the bases are adenine, cytosine, guanine, and uracil. In the present disclosure, the term “siRNA” and “shRNA” refer to a class of double-stranded RNA molecules that operate using the concept of RNA interference (RNAi). The difference between siRNA and shRNA is their secondary structure, as shRNAs are so named for the presence of tight hairpin turns in their secondary structure.

[0041] Thus, in one example, the nucleic acid sequence disclosed herein is a short interfering RNA (siRNA) sequence or a short hairpin RNA (shRNA) sequence. In another example, the nucleic acid sequence is siRNA. In yet another example, the nucleic acid sequence is shRNA.

[0042] In one example, the siRNA sequence is between 15 to 150 base pairs, between 60 to 100 base pairs, between 70 to 120 base pairs, about 60 base pairs, about 65 base pairs, about 70 base pairs, about 75 base pairs, about 80 base pairs, about 85 base pairs, about 90 base pairs, about 95 base pairs, about 100 base pairs, about 105 base pairs, or about 110 base pairs in length. In another example, the siRNA sequence is at least 15 base pairs, at least 20 base pairs, at least 25 base pairs, at least 30 base pairs, at least 35 base pairs, at least 40 base pairs, at least 45 base pairs, or at least 50 base pairs in length.

[0043] In another example, the shRNA sequence comprises stems with the length of between 15 to 30 base pairs, between 19 to 29 base pairs, between 15 to 20 base pairs, between 20 to 30 base pairs, about 18 base pairs, about 19 base pairs, about 20 base pairs,

about 21 base pairs, about 22 base pairs, about 23 base pairs, about 24 base pairs, about 25 base pairs, about 26 base pairs, about 27 base pairs, about 28 base pairs, about 29 base pairs, or about 30 base pairs.

[0044] In yet another example, the nucleic acid sequence disclosed comprises one of the sequences of SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 36, SEQ ID NO. 37, SEQ ID NO. 38, SEQ ID NO. 39, SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 11, SEQ ID NO. 44, SEQ ID NO. 12, SEQ ID NO. 13, SEQ ID NO. 15, SEQ ID NO. 45, SEQ ID NO. 46, SEQ ID NO. 16, SEQ ID NO. 17, SEQ ID NO. 19, SEQ ID NO. 47, SEQ ID NO. 20, SEQ ID NO. 21, SEQ ID NO. 26, SEQ ID NO. 27, SEQ ID NO. 28, SEQ ID NO. 29, SEQ ID NO. 30, SEQ ID NO. 31, SEQ ID NO. 32, or SEQ ID NO. 33.

[0045] Collectively, the data shown herein, for example in Fig. 2A-D as disclosed herein, shows that it is possible to generate siRNAs reproducibly that are highly specific and selective for single nucleotide changes, with extensive screening. Thus, in one example, the nucleic acid sequence disclosed herein comprises one of the sequences of SEQ ID NO. 9, SEQ ID NO. 13, SEQ ID NO. 16, SEQ ID NO. 17, or SEQ ID NO. 21.

[0046] In one example, the nucleic acid sequence disclosed herein comprises one of the sequences of SEQ ID NO. 8 (R175H Si-1-R175H), SEQ ID NO. 9 (R175H Si-2-R175H), SEQ ID NO. 12 (R248W/Q Si-3-R248W/R248Q), SEQ ID NO. 13 (R248W/Q Si-4-R248W/R248Q), SEQ ID NO. 16 (R249S/M/G Si-5-R249S/R249M/R249G), SEQ ID NO. 17 (R249S/M/G Si-6-R249S/R249M/R249G), SEQ ID NO. 20 (R273H/L Si-7-R273H/R273L), or SEQ ID NO. 21 (R273H/L Si-8-R273H/R273L).

[0047] In yet another example, nucleic acid sequence disclosed herein comprises one of the sequences of SEQ ID NO. 26, SEQ ID NO. 27, SEQ ID NO. 28, SEQ ID NO. 29, SEQ ID NO. 30, SEQ ID NO. 31, SEQ ID NO. 32, or SEQ ID NO. 33.

[0048] In another example, the nucleic acid sequence disclosed herein comprises one of the sequence pairs of SEQ ID NO. 26 and SEQ ID NO. 27; SEQ ID NO. 28 and SEQ ID NO. 29; SEQ ID NO. 30 and SEQ ID NO. 31; or SEQ ID NO. 32 and SEQ ID NO. 33.

[0049] In a further example, in one example, the nucleic acid sequence disclosed herein comprises one of the sequences of SEQ ID NO. 9, SEQ ID NO. 13, SEQ ID NO. 16, SEQ ID NO. 17, or SEQ ID NO. 21.

[0050] In one example, the nucleic acid sequence is SEQ ID NO. 2. In another example, the nucleic acid sequence is SEQ ID NO. 3. In one example, the nucleic acid sequence is SEQ ID NO. 4. In one example, the nucleic acid sequence is SEQ ID NO. 5. In one example,

the nucleic acid sequence is SEQ ID NO. 36. In one example, the nucleic acid sequence is SEQ ID NO. 37. In one example, the nucleic acid sequence is SEQ ID NO. 38. In one example, the nucleic acid sequence is SEQ ID NO. 39. In one example, the nucleic acid sequence is SEQ ID NO. 7. In one example, the nucleic acid sequence is SEQ ID NO. 8. In one example, the nucleic acid sequence is SEQ ID NO. 9. In one example, the nucleic acid sequence is SEQ ID NO. 11. In one example, the nucleic acid sequence is SEQ ID NO. 44. In one example, the nucleic acid sequence is SEQ ID NO. 12. In one example, the nucleic acid sequence is SEQ ID NO. 13. In one example, the nucleic acid sequence is SEQ ID NO. 15. In one example, the nucleic acid sequence is SEQ ID NO. 45. In one example, the nucleic acid sequence is SEQ ID NO. 46. In one example, the nucleic acid sequence is SEQ ID NO. 16. In one example, the nucleic acid sequence is SEQ ID NO. 17. In one example, the nucleic acid sequence is SEQ ID NO. 19. In one example, the nucleic acid sequence is SEQ ID NO. 47. In one example, the nucleic acid sequence is SEQ ID NO. 20. In one example, the nucleic acid sequence is SEQ ID NO. 21. In one example, the nucleic acid sequence is SEQ ID NO. 26. In one example, the nucleic acid sequence is SEQ ID NO. 27. In one example, the nucleic acid sequence is SEQ ID NO. 28. In one example, the nucleic acid sequence is SEQ ID NO. 29. In one example, the nucleic acid sequence is SEQ ID NO. 30. In one example, the nucleic acid sequence is SEQ ID NO. 31. In one example, the nucleic acid sequence is SEQ ID NO. 32. In one example, the nucleic acid sequence is SEQ ID NO. 33.

[0051] In one example, the nucleic acid sequence comprises SEQ ID NO. 24 (AAGCTTT), SEQ ID NO. 40 (TTCAAGAGA) and SEQ ID NO. 41 (TTTTTTA), whereby the nucleic acid sequence has the following structure: 5'-AAGCTTTN₍₁₉₋₂₉₎(sense sequence)TTCAAGAGAN₍₁₉₋₂₉₎(antisense sequence)TTTTTTA-3'. This is an exemplary shRNA upper oligonucleotide, whereby (other than the siRNA sequence which is referred to as N₍₁₉₋₂₉₎) the nucleotides indicated at the front and end of each oligonucleotide are for the restriction enzyme cutting site. The middle sequence (in this example TTCAAGAGA) is for the formation of a stem loop.

[0052] In another example, the nucleic acid sequence comprises SEQ ID NO. 25 (AGCTTAAAAA), SEQ ID NO. 42 (TCTCTTGAA) and SEQ ID NO. 43 (GGG), whereby the nucleic acid sequence has the following structure: 5'-AGCTTAAAAAN₍₁₉₋₂₉₎(sense sequence)TCTCTTGAAN₍₁₉₋₂₉₎(antisense sequence)GGG-3'. This is an exemplary shRNA lower oligonucleotide, whereby (other than the siRNA sequence which is referred to as N₍₁₉₋₂₉₎) the nucleotides indicated at the front and end of each oligonucleotide are for the

restriction enzyme cutting site. The middle sequence (in this example TCTCTTGAA) is for the formation of a stem loop.

[0053] The results presented here demonstrate that siRNAs that are highly-specific and capable of distinguishing one nucleotide change can indeed be regularly generated, and highlight their utility in targeting four p53 hot-spot mutants. These four p53 mutants account for about 20% of all p53 mutations found in cancers, and thus represent the possibility of targeting about 10% of all cancers. Targeting mutant p53 resulted in improved chemosensitivity, as it had no effects on the wild-type p53 protein in the heterozygous cells, allowing it to function to induce cell death. Furthermore, abrogation of mutant p53 expression in cancer cells expressing only mutant p53, as often seen in later stages of cancers where the wild-type p53 allele is lost due to loss-of-heterozygosity, resulted in retardation of tumour growth *in vivo* even when used as a mono-therapy. This data highlights the therapeutic use of these p53 mutant-specific siRNAs, whose effects could be further enhanced in combination with other chemotherapeutic agents or radiotherapy. Hence, these data provide the impetus to target mutant p53 directly for clinical benefit, which could be translated to the clinical settings soon.

[0054] Mutant p53 were chosen to demonstrate the nucleotide-specific siRNAs, as it is the most mutated gene across all cancers, and importantly, not all mutants behave similarly, thus, requiring selective agents to target each of them. Moreover, targeting mutant p53 represents a huge untapped route to retard tumour cell growth and metastasis, and to improve sensitivity to general cytotoxic agents, and would therefore find applicability against most cancer types. Similarly, targeting other driver oncogenes with specific siRNAs in conjunction with mutant p53 is thought to enhance the therapeutic effects, and therefore, use of a cocktail of siRNAs against the major genetic alterations in each cancer type is also possible in a clinical setting.

[0055] Thus, in one example, there is disclosed a method of treating cancer in a subject. In another example, the method comprises administering to the subject one or more nucleic acid sequences as described in the present application. In yet another example, the nucleic acid sequences target one or more point mutations within a target gene. In another example, the target gene is one or more tumour suppressor genes. In yet another example, the method comprises administering to the subject one or more nucleic acid sequences as disclosed herein, wherein the nucleic acid sequences target one or more point mutations within a target gene, wherein the target gene is a tumour suppressor gene. Also disclosed herein is use of one or more nucleic acid sequences as disclosed herein in the manufacture of a medicament for treating cancer in a subject. Further disclosed herein is the use of one or more of the nucleic

acid sequences disclosed herein in therapy. In another example, the nucleic acid sequences disclosed herein are for use in therapy.

[0056] The term "treat" or "treating" as used herein is intended to refer to providing a pharmaceutically or therapeutically effective amount of, for example, a nucleic acid, a protein, or a respective pharmaceutical composition or medicament thereof, sufficient to act prophylactically to prevent the development of a weakened and/or unhealthy state; and/or providing a subject with a sufficient amount of the pharmaceutical composition or medicament thereof so as to alleviate or eliminate a disease state and/or the symptoms of a disease state, and a weakened and/or unhealthy state. As is known in the art, the pharmaceutically effective amount of a given composition will also depend on the administration route. In general the required amount will be higher, if the administration is through, for example, the gastrointestinal tract (e.g. by suppository, rectal, or by an intragastric probe), and lower if the route of administration is parenteral, e.g. intravenously.

[0057] The generation and characterization of siRNAs that are highly specific for the various p53 mutants that are highly represented in human cancers has been shown. This data translates directly for clinical evaluation with the appropriate delivery mechanisms.

[0058] In one example, administration of the one or more of the nucleic acid sequences results in one or more of the following effects, including but not limited to, cell death, abrogation of addiction to any one or more of the target genes, a dominant negative effect, increased sensitivity to one or more anti-cancer agents, and retardation or halting of tumour growth. In another example, the nucleic acid sequences disclosed are administered with a therapeutic agent.

[0059] As used herein, the term "therapeutic agent" refers to a chemical compound or composition capable of inducing a desired therapeutic effect when properly administered to a subject. For example, an anti-diabetic agent is considered a therapeutic agent, in the sense that it is administered to treat, for example, diabetes in a subject. Thus, in one example, the method disclosed herein comprises administration of a therapeutic agent. In another example, the therapeutic agent is an anti-cancer agent. In another example, the anti-cancer agent is selected from the group consisting of 10-hydroxycamptothecin, abraxane, acediasulfone, aclarubicine, aklavine hydrochloride, ambazone, amsacrine, aminoglutethimide, anastrozole, ancitabine hydrochloride, L-asparaginase, azathioprine, bleomycin, bortezomib, busulfan, calcium folinate, carboplatin, carpecitabine, carmustine, celecoxib, chlorambucil, cisplatin, cladribine, colchicine, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, dapsone, daunorubicin, dibrompropamide, diethylstilbestrol, docetaxel, doxorubicin, emetine,

[0060] Thus, the methods disclosed herein can be used to treat a hyperproliferative disease, for example, cancer. In one example, the cancer is found to be in, or originates from, organs and areas of a mammal body, including, but not limited to the oesophagus, upper respiratory tract, skin, epithelial, central nervous system, ovarian, breast, gastro-intestinal, large intestines, small intestines, colorectal, liver, adenocarcinoma, adrenal adenocarcinoma, thyroid, lung, pancreas, kidney, endometrial, hematopoietic, muscles, connective tissue (such as tendon or cartilage), bone, soft tissue, lymphoid tissue, lymph and the immune system. In another example, the type of cancer is, but is not limited to, melanomas, myelomas, carcinomas, sarcomas, lymphomas, blastomas and germ cell tumours. In another example, the cancer is, but is not limited to, lung carcinoma, malignant melanoma, colon carcinoma, breast carcinoma, endometrial adenocarcinoma, rhabdomyosarcoma, kidney adenocarcinoma, colon adenocarcinoma, hepatocellular carcinoma, bronchial squamous cancer, ovarian carcinoma and pancreatic adenocarcinoma.

[0061] In another example, the cancer is a cancer cell line including, but not limited to, A549, A375, HCT116, RKO, AU565, SKBR3, HCC1395, HEC 1A, RD, 786-O, COLO-320DM, PLC-PRF/5, KNS-62, BT549, ASPC1, WiDR1 and H1975. In another example, the cancer is dependent on one or more of the tumour suppressor genes. In yet another example, the tumour suppressor gene is p53. In a further example, the cancer is dependent on the tumour suppressor gene, wherein the tumour suppressor gene is p53.

[0062] The results presented herein demonstrate that siRNAs that are specific and capable of distinguishing one nucleotide change can indeed be regularly generated, and highlight their utility in targeting four p53 hot-spot mutants. The four p53 mutants disclosed herein account for about 20% of all p53 mutations found in cancers, and targeting them represents the possibility of targeting about 10% of all cancers. Targeting mutant p53 resulted in improved chemo-sensitivity, as it had negligible or no effects on the wild-type p53 protein in the heterozygous cells, allowing the latter to function to induce cell death. Furthermore, abrogation of mutant p53 expression in cancer cells expressing only mutant p53, as often seen in later stages of cancers where the wild-type p53 allele is lost due to loss-of-heterozygosity, resulted in retardation of tumour growth *in vivo* even when used as a monotherapy. This data highlights the therapeutic potential provided by the RNA constructs as disclosed herein, whose effects could be further enhanced in combination with other chemotherapeutic agents or radiotherapy. Hence, the data shown herein also demonstrates that targeting mutant p53 directly has clinical benefits and would translate into a clinical setting.

[0063] RNAs, for example siRNAs, have been generated successfully to silence gene expression and has been extensively used in research, and also been translated to the clinical setting. Most of these siRNAs target the whole gene (protein), without cross-reactivity to other related genes. However, only few examples exist for the generation of siRNAs that are capable of discerning single nucleotide changes found in the disease states. The ones that have been generated with some specificity for single nucleotides include those against R248W mutant p53. These have been shown to be relatively specific in reporter assays and in overexpression systems, though some level of cross-reactivity with the wild-type protein is often noted. Moreover, many of the siRNA have not been tested in a large number of cell lines to establish their specificity unequivocally. These factors highlight the enormous challenges in obtaining siRNAs that show specificity at the nucleotide level, and which can be used on critical genes that affect a multitude of process in normal physiology, like p53. The data shown herein has revealed that a large library of siRNAs has to be tested prior to obtaining highly specific ones, especially since the effects of the addition or subtraction of a few nucleotides in the siRNA sequences can make a huge difference. Very subtle changes in the sequences of the siRNA significantly can affect the specificity and lead to marked differences in selectivity, and highlights that one cannot intuitively predict the effects of the various sequences. Though it is relatively possible to obtain siRNAs that appear to be nucleotide specific, especially when assayed against one or two cell lines or using transfection systems, analyses against a large panel of cellular systems is essential to ensure that they are specific. This is crucial when these siRNAs are intended for use in the clinical setting. The set of siRNA/shRNA sequences presented herein represents a unique set of RNAs that are capable of specifically targeting almost 20% of all cancers with mutations in p53, supporting the notion that with sufficient screening, nucleotide-specific siRNAs/shRNAs can be generated and evaluated in clinical trials.

[0064] The mutant p53 has been chosen to demonstrate the ability to generate nucleotide-specific siRNAs, as it is the most mutated gene across all cancers. Importantly, not all p53 mutants behave similarly, and thus, targeting mutant p53 requires selective agents to target each of them individually. Moreover, targeting mutant p53 represents an untapped route to retard tumour cell growth and metastasis, and to improve sensitivity to general cytotoxic agents, and would therefore find applicability against most cancer types. As highlighted earlier, mutant p53 can exist either with the wild-type allele in the earlier stages of tumorigenesis, or by itself after the loss of the wild-type allele due to LOH in later stages. In the earlier stages, mutant p53 inhibits the WT protein through the dominant-negative (DN)

effect, and at the later stages, the mutant provides a survival advantage independent of the wild-type allele. The data shown herein demonstrates that the mutant p53-specific siRNAs are capable of relieving both the dominant-negative (DN) effect, as well as the addiction of cancer cells to mutant p53, and can therefore be used widely as long as the mutation is present in the tumours. Similarly, targeting other driver oncogenes with specific siRNAs in conjunction with mutant p53 is understood to enhance the therapeutic effects, and it is thought that a cocktail of siRNAs (or other RNA capable of silencing target gene expression) against the major genetic alterations in each cancer type will be clinically beneficial, with the aim of minimizing cross-reactivity, and thus, reducing side effects associated with many of today's cancer drugs.

[0065] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0066] As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a genetic marker" includes a plurality of genetic markers, including mixtures and combinations thereof.

[0067] As used herein, the term "about", in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

[0068] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have

specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0069] Certain embodiments may also be described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0070] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0071] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

Materials and Methods

Cell culture

[0072] Cell lines were obtained from ATCC and JCRB and were cultured under standard conditions (37°C, 5% CO₂) with the following media: DMEM with 4.5 g/L glucose and 10% FBS (Hyclone) for H1299, RKO, HCT116, A549, A375, SKBR3, RD, PLC-PRF-5, KNS-62 and HEC1A cell lines; RPMI-1640 and 10% FBS (Hyclone) for AU565, HCC1395, COLO-320DM, 786-O, ASPC-1, WiDR and H1975; RPMI-1640 with 0.023 IU/ml insulin and 10% FBS (Hyclone) for BT-549; RKO p53^{+/-} and ^{+/R248W} and HCT p53^{+/-} and ^{+/R248W}.

siRNA design

[0073] A large library of siRNAs were designed to target p53 hot-spot mutations (R175H, R248W, R249S and R273H), and from these, 8 were shortlisted for the four mutants for further characterization (si-1-8). An siRNA against all p53 alleles generated in our screen was used as a positive control for pan-p53 targeting. Control scrambled siRNA had no bio-

informatically predicted sequence target in the human genome and was used as a negative control.

Transfection of p53 siRNA/shRNA, and RNA and protein analyses

[0074] 2.5×10^5 cells per well were seeded in a 6-well plate 24 hours before transfection. The cells were transfected with 80 nM siRNA or 1 μ g of the pRetroSuper-shRNAs using LipofectamineTM 2000 reagent (Invitrogen) as per the manufacturer's description. Each transfection was performed in triplicate and the cells were harvested with 1mL of TRIzol reagent (Invitrogen) 72 hours after transfection. For co-transfection with p53 cDNAs, the latter were transfected 24 hours after the siRNA transfection and cells were analysed 48 post cDNA transfection (i.e. 72 hours post-siRNA transfection).

[0075] Total RNA isolation was performed using Invitrogen's standard protocol, and cDNA was prepared using Superscript II reverse transcription (Invitrogen). Quantitative and semi-quantitative reverse transcriptase (RT)-PCR analysis was performed on the following p53 target genes: p21, pig3, mdm2, noxa and gapdh, as described.

[0076] Cell extracts were prepared in lysis buffer (0.7% NP40; Tris.Cl, pH 7.4; 70 mM EDTA; 200 mM NaCl on ice for 10 minutes). After protein quantitation, 30-50 μ g of lysate was loaded on SDS-polycrylamide gel (12%) electrophoresis (SDS-PAGE), and the resolved proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Breda, The Netherlands). The detection of the protein was done with ECL (GE Healthcare, Waukesha, WI, USA). p53 was detected with a mouse anti-p53 monoclonal antibody (DO-1 from Santa Cruz Biotechnology, #SC126) and actin was detected with a rabbit anti-actin antibody (Sigma, #82061). Parallel gels were run with equal amounts of lysates and probed with the various antibodies separately, in cases where background from the first antibody was high. Quantification of western blots was done using the ImageJ software by lane plotting and peak labelling (signal intensity quantification). For each sample, the ratio of p53 to Actin band intensity was calculated and normalized to the ratio of si-scr/sh-scr control. Values represent normalized fold change.

Cell death assays

[0077] Cells were transfected with 80 nM siRNA and harvested 72 hours post-transfection, including floating cells in the medium. Cells were washed 2X in PBS and were fixed in 70% ethanol overnight and were treated with RNase for 20 minutes before addition of 5 μ g/ml propidium iodide (PI) and flow cytometric analysis by flow cytometry (BD Biosciences FACScalibur), to measure apoptosis (sub-G1 DNA content).

Design of shRNA template oligonucleotides and construction of plasmid

[0078] shRNA target sequences were designed to be homologous to the siRNA sequences afore-described. The pRetro-Super vector contains a human H1 polymerase-III (pol-III) promoter for shRNA expression. Each shRNA insert was designed as a synthetic duplex with overhanging ends identical to those created by restriction enzyme (RE) digestion (*Bam*HI at the 5' and *Hind*III at the 3'). The coding region for each hairpin is nested within a single oligonucleotide (upper oligonucleotide: 5'-AAGCTTTN₍₁₉₋₂₉₎(sense sequence)TTCAAGAGAN₍₁₉₋₂₉₎(antisense sequence)TTTTTTA-3') and its complementary equivalent (lower oligonucleotide: 5'-AGCTTAAAAAN₍₁₉₋₂₉₎(sense sequence)TCTCTTGAAN₍₁₉₋₂₉₎(antisense sequence)GGG-3'). These ranged in size from 60 - 100 bases (for hairpins with 19 - 29 bp stems). Each duplex contained a transcription initiation base, the shRNA encoding region (sense stem, loop sequence and anti-sense stem), a termination spacer and a pol-III termination signal consisting of a run of at least 4 'T's. The transcription initiation base was an 'A' or 'G' (required for efficient pol-III transcription initiation) and was only included if the first base of the hairpin stem was not a purine. The termination spacer was any base but 'T' and was included only if the last base of the anti-sense stem was 'T' so as to prevent premature termination via an early run of 'T's. Oligonucleotides were ordered at the minimal synthesis and purification scales (0.05µM and desalt, Sigma-Aldrich). Each oligonucleotide was re-suspended in water at a 100µM concentration and 10µl from each was added to 20µl of 2X annealing buffer (200mM Potassium acetate, 60mM HEPES KOH pH 7.4, 4mM Mg-acetate), heated to 95°C for 10 minutes, slowly equilibrated to room temperature and diluted 1:1000 fold for ligation. The insert and vector were ligated, and transformed into TOP10 or DH5α competent cells. Clones with the shRNA insert were selected and purified before transfection.

Colony formation assay

[0079] The indicated cell lines were transfected with the indicated shRNA plasmids containing oligonucleotide sequences for silencing the various mutant p53 and were selected for 2 weeks on 15µg/ml of blasticidine (Sigma, USA). Colonies were stained with crystal violet solution (Merck), as described.

Generation of shRNA expressing cell lines for in vivo tumour growth analysis

[0080] Viruses for p53 mutant-specific shRNAs were produced using pCL-Ampho amphotropic virus packaging plasmid in HEK293T cells. Briefly, retroviruses were prepared by transfection of HEK293T cells with the 1.5µg of the appropriate shRNA and 1.0µg of the packaging plasmid using lipofectamine 2000™. Retroviral supernatants were harvested at 24h after transfection, filtered through 0.45µM syringe filter, aliquoted and flash frozen.

3.5ml of retroviral supernatant was used to transduce 5×10^5 cells in a 10cm dish in the presence of 8µg/ml of polybrene (Sigma) in triplicates in 6cm dish. A second transduction was performed the following day. The cells were selected using 10µg/ml of blasticidine for 48h after the second transduction, and harvested for *in vivo* xenograft studies. Parallel cultures were used for immunoblots analysis to assess the efficiency of p53 knockdown.

[0081] Cell lines expressing the respective shRNA were harvested, and mixed with 50% Matrigel on ice (Corning®Matrigel® basement membrane matrix) (Sigma), and subcutaneously injected in the right flank of female C.B-17 SCID mice (6-8 weeks of age), and cells transduced with the scrambled shRNA were injected on the left flanks of each mice. Tumour volume was assessed with a caliper twice per week and values were taken down as soon as tumours became palpable. Calculation of tumour volume was done according to $V=1/2*(length*width^2)$. Values are plotted as means with standard deviation. Statistical significance between grow curves was calculated with PRISM software (GraphPad Prism Software Inc., San Diego, CA) using unpaired (two-tailed) t-test. Four-five mice were used for each treatment, in each group.

[0082] When mice were sacrificed, tumour tissues were excised and fixed in 10% formalin over-night, dehydrated and embedded in paraffin and 5µm sections were prepared. Anti-p53 staining was done using p53 1C12 Mouse monoclonal antibody (Cell Signaling Technology, #2524) with a concentration of 1:1500. Staining signal was developed using Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (#5007). All the animal experiments were conducted as approved by the Institution's Animal Care and Ethics Committee.

Results

Design and selection of allele-specific siRNAs for hot-spot p53 mutants

[0083] Starting point for the generation of siRNAs was that these siRNA will be capable of only silencing the mutant p53 alleles, without having an impact on WT p53 expression. To this end, a library of a large number of siRNAs was generated by performing sequence walks, such that the position of the mutant nucleotide was varied with respect to the entire siRNA strand. All the siRNAs were transfected in a series of H1299-based isogenic cell lines which stably expressed the various p53 mutants, or the temperature-sensitive (TS) WT p53, and data from representative siRNAs that show specific activity against for the four hot-spot mutants: R175H, R248W, R249S and R273H are shown (Fig. 1A). These were among the few shortlisted siRNAs that had specific activity for the respective p53 mutants. All the indicated

siRNAs were transiently transfected in all the isogenic cell lines, which were harvested for analysis of the p53 protein expression 24 hours later, by immunoblotting. As shown in Fig. 1B, si-p53, which targets all p53 indiscriminately, was capable of reducing the expression of p53 in all cell lines, compared to scrambled siRNA or cells that were not transfected (last 3 lanes on the right of the gel images). Most of the mutation-specific siRNAs showed specificity and were able to discriminate the intended mutants, with minimal to negligible effects on other mutants or the WT p53: for instance, si-1 and si-2, which are specific for R175H mutant p53, were capable of reducing R175H expression, but had minimal impact on the other p53 mutants and WT p53. Similarly, si-3 and si-4 which are specific for R248W mutant p53 were capable of markedly reducing the expression of R248W mutant, without impacting other mutants. On the other hand, however, si-3 which also targets the R248W mutant, though capable of reducing the expression of its intended mutation, also led to a decrease in the expression of WT p53. Similarly, while si-8 which targets R273H was very specific, si-7 also had some effects on both WT p53 and the R249S mutant. This data indicates that evaluation of multiple siRNAs generated against the same mutation on multiple cell systems is crucial to obtain highly mutant-specific reagents.

Mutation-specific siRNA-mediated silencing of endogenous mutant p53 expression

[0084] The efficacy and specificity of the selected siRNAs were evaluated on a panel of 17 different cancer cell lines that express either WT or the various mutant p53 (Table 1). Similar to the H1299 isogenic cell lines, these cells were transfected with the specific siRNAs or the positive control si-p53 which indiscriminately suppresses the expression of both WT and mutant p53 (Fig. 2A-D). As noted earlier with the H1299-isogenic cell settings, the si-2 was able to specifically down-regulate the expression of the R175H mutant in cells expressing this mutant (i.e. HCC1395, SKBR3 and AU565), without having an impact on the expression of WT p53 in three cell lines (i.e. HCT116, A549 and A375) (Fig. 2A). Similarly, si-4, which is specific for the R248W mutant p53, efficiently inhibited p53 expression in COLO-320DM, 786-O and RD cells expressing the R248W mutant (Fig. 2B), with no appreciable impact on WT p53 expression in the other cell lines. Similar results were obtained with si-6 which is selective for the R249S mutant (in BT549, KNS-62 and PLC-PRF5 cells), and si-8 which is specific for R273H in R273H-expressing ASPC1, H1975 and WIDR cells (Fig. 2C and D). The other siRNAs against the specific mutants, si-1, si-3, si-5 and si-7, were also specific for the intended mutants, occasionally displaying slight effects on WT p53. Therefore, the specificity of each of the mutant p53-specific siRNAs was also evaluated on various other mutant p53-expressing cells. As shown in Fig. 8, si-2, si-4, si-6

and si-8 were highly specific and did not affect the expression of the other mutant p53 in all cell lines tested. However, and as noted earlier on the H1299-isogenic cell system, si-1, si-3, si-5 and si-7 had occasional impact on other mutants in some cell lines. It is noteworthy that si-3 against R248W is similar to the siRNA published to target this specific mutation (Martinez, L.A., Naguibneva, I., Lehrmann, H., Vervisch, A., Tchénio, T., Lozano, G., and Harel-Bellan, A. (2002). Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways. PNAS; 99; 14849–14854). However, extensive analysis indicates that while the siRNA as published in Martinez et al. indeed targets R248W, it also has some non-specific activity against WT and the R175H mutant in some cell lines. This demonstrates that very subtle changes in the sequences of the siRNA significantly affects the specificity and makes a marked difference in specificity between different sequences, and highlights that one cannot intuitively predict the effects of the various sequences. Collectively, these results show that it is possible to generate siRNAs reproducibly that are highly specific and selective for single nucleotide changes, with extensive screening. Based on these analyses, si-2 (for R175H mutant); si-4 (for R248W mutant); si-5 and si-6 for (R249S mutant) and si-8 (for R273H mutant) were shortlisted for further in-depth characterization. Thus, in one example, the nucleic acid sequence disclosed herein comprises one of the sequences of SEQ ID NO. 9, SEQ ID NO. 13, SEQ ID NO. 16, SEQ ID NO. 17, or SEQ ID NO. 21.

Allele-specific knock-down of mutant p53 expression promotes apoptosis and induces p53-target gene expression

[0085] As tumour cells expressing mutant p53 have been shown to be addicted to its presence for survival, it was first evaluated if the mutation-specific siRNAs will be able to alleviate this phenomenon and induce cell death in the respective mutant-expressing cancer cell lines. Transfection of the specific siRNAs in the respective mutant p53-expressing cell lines universally led to increased apoptosis, as determined by the percentage of sub-G1 population (Fig. 3). While untransfected and scrambled siRNA transfected cells gave basal death, transfection of either the pan-p53 siRNA, or the specific mutant p53 siRNAs led to increased cell death in the cell lines expressing the respective mutant p53 (% sub-G1 population in si-scr vs. si-p53 vs. si-mutant p53 → AU565: 26.7 vs. 39.6 vs. 36.6; 786-O: 18.0 vs. 37.2 vs. 31.7; BT549: 10.4 vs. 39.6 vs. 32.9; H1975: 11.5 vs. 25.8 vs. 28.5) (Fig. 3A). Importantly, si-p53 reduced cell death in WT p53-expressing HCT116 cells (si-scr vs. si-p53: 7.6 vs. 2.1), confirming that silencing of mutant p53 expression by a generic p53 siRNA or the mutation-specific siRNA leads to enhanced cell death only in mutant

p53-expressing cancer cell lines. Cross evaluation of the siRNAs on cancer cells expressing other p53 mutants also confirmed their specificity in silencing only the intended mutants, but not others (Fig. 9). Concurrent treatment of these cells with the chemotherapeutic agent cisplatin (CDDP) potentiated the cell death induced by the mutation-specific siRNA only in mutant p53-expressing cancer cell lines, but not in WT p53-expressing HCT116 cells (Fig. 10). Together, these data indicate that cell death induced by silencing mutant p53 further synergizes with cytotoxic drug treatment.

[0086] It had been previously shown that silencing of mutant p53 in mutant p53-expressing cell lines led to induction of the expression of canonical p53-target genes, concomitant to the attenuation of the addiction to mutant p53 for survival. It was therefore evaluated if this phenomenon also occurs in the context of mutant p53-specific siRNA treatment. To this end, quantitative RT-PCR (qPCR) was performed for several p53 target genes such as p21, Mdm2, Noxa and Pig3 (Fig. 4). Expression of mRNAs for all the tested p53 target genes was significantly down-regulated following p53 down-regulation in WT p53 expressing HCT116 cells, as expected, but were minimally altered by the mutant-specific siRNAs in these cells (Fig. 4A). By contrast, transfection of the mutant-specific siRNAs (i.e. si-2, si-4, si-6 and si-8) or the general p53 siRNA in mutant p53-expressing cells lines led to a significant up-regulation of almost all the target genes tested (Fig. 4B). Similar results were obtained using the mutant-specific siRNAs on a different set of cell lines expressing the corresponding mutants (Fig. 11). Moreover, as noted with the cell viability experiments, concurrent treatment of cells with mutant p53-specific siRNAs with CDDP led to enhanced induction of p53 target genes, highlighting synergy. Furthermore, inhibition of p53 expression in WT p53 expressing cells treated with CDDP led to the expected reduction in target gene expression, indicating the specificity of the effect of the mutant p53 siRNAs on mutant p53-expressing cell lines.

Inhibition of mutant p53 expression using mutant p53-specific shRNA expression vectors

[0087] To evaluate the long-term effects of the mutant p53-specific silencing, short-hairpin RNAs that express the mutant p53 specific sequences from the si-2, si-4, si-6 and si-8 siRNAs, as well as the general p53-specific siRNA, were generated using the pSuper vector. Initial tests evaluating their efficacy in silencing the expression of the specific mutant p53 were performed in the respective mutant p53-expressing cells lines, after transient transfection of the plasmids. Immunoblot analyses indicated that the mutant p53-specific shRNAs were equally potent in suppressing the expression of the intended mutant p53 in the respective cell lines, unlike the control scrambled shRNA (Fig. 5A). Based on this, the effects

of suppressing mutant p53 expression on long-term colony growth was evaluated, which again confirmed that cellular growth was significantly inhibited by silencing the respective mutant p53 (Fig. 5B). Similar results were obtained in short-term apoptosis assays (Fig. 12A), indicating that shRNA-based mutant p53 silencing is equally effective in promoting cell death of mutant p53 expressing cancer cells.

[0088] It was also evaluated whether the mutation-specific shRNAs are capable of silencing various mutants that occur at the same nucleotide position on p53. To test this hypothesis, the HEC-1A cancer cell line which expresses the R248Q mutation was utilised, and transfected the sh-4 which was initially generated against the R248W mutation. As shown in Fig. 12B-D, sh-4 was capable of silencing the expression of the R248Q p53 mutant, which concomitantly led to increased cell death in short and long-term assays. This data suggests that mutation-specific si/shRNA against a particular mutated nucleotide residue is specific for the residue at that position, but does not necessarily discriminate the substituted residues, and hence, in one example, can be widely used for the many mutations found at a particular nucleotide position, especially in the case of mutant p53.

Relief of dominant-negative effect of mutant p53 and enhancement of cell death upon mutant p53 silencing

[0089] While expression of mutant p53 alone results in addiction of cancer cells to the mutant protein for survival, co-expression of both WT and mutant p53 in the heterozygous state leads to a dominant-negative (DN) effect of the mutant protein over the WT protein, leading to amelioration of the latter's functions in target gene activation and apoptosis induction. It had been previously shown that reducing the mutant p53 levels in this heterozygous context leads to restoration of WT p53 function, and sensitizes cells to chemotherapeutic agents and irradiation. Hence, the mutant p53-specific shRNAs were evaluated for their use in reducing mutant p53 levels in mutant heterozygous cells, to improve therapeutic response. To this end, two sets of isogenic colorectal cells lines (RKO and HCT116), which are heterozygous for p53 (p53+/-) or heterozygous for mutant p53 (p53+/R248W), were utilised. Transfection of sh-4 which is specific for the R248W mutant led to a significant decrease of total p53 in the p53+/R248W cells but not in p53+/- HCT and RKO cells, indicating specificity (Fig. 6A and Fig. 13A). Concomitant analysis of long-term survival revealed that the sh-4 transfected p53+/R248W cells were more prone to growth inhibition compared to the p53+/- cells (Fig. 6B and Fig. 13B). Moreover, p53 target gene induction was significantly induced only in the p53+/R248W cells compared to the p53+/- cells when sh-4 was transfected (Fig. 6C and Fig. 13C), collectively indicating that

suppression of mutant p53 relieves the DN effect, and leads to elevated cell death in mutant p53-expressing cells.

[0090] The effects of these siRNAs on cell death upon cisplatin (CDDP) treatment was also analysed, which indicated that the presence of mutant p53 reduced cell death (% sub-G1 cells in RKO p53+/- vs. p53+/R248W cells in untransfected and scrambled shRNA transfected: 50.9 and 50.3 vs. 14.9 and 11.3; in HCT cells: 61.1 and 51.2 vs. 32.1 and 28.6), highlighting the DN effects (Fig. 6D-E and Fig. 13D-E). By contrast, transfection of mutant-specific sh-4 led to a significant increase in cell death particularly in the p53+/R248W cells compared to the p53+/- cells (% sub-G1 cells in RKO p53+/- cells, untransfected vs. sh-4 shRNA: 50.9 vs. 49.7; in RKO p53+/R248W cells: 14.9 vs. 86.1; in HCT p53+/- cells: 61.1 vs. 68.8; in HCT p53+/R248W cells: 32.1 vs. 66.9; Fig. 13E). This data together demonstrates that silencing mutant p53 specifically without impacting WT p53 expression leads to relief of DN effects and sensitizes mutant-p53 expressing cells to death, which is enhanced by chemotherapeutic drug treatment.

Therapeutic targeting of mutant p53 retards tumour growth in vivo

[0091] Finally, it was evaluated whether the mutant p53-specific si/shRNAs would be effective in retarding tumour growth *in vivo*, by using the cell-based xenograft model to monitor the growth of cancer cell lines (RD, PLC-PR5 and H1975) expressing the scrambled or the respective mutant-specific shRNAs. Cancer cells which express the various p53 mutants and transiently infected with viral particles expressing the scrambled shRNA grew to a large volume over time, whereas the cells expressing the respective mutant p53-specific shRNAs were markedly retarded in growth *in vivo* (Fig. 7A). Histological analysis of tumours at sacrifice revealed that the mutant-specific shRNA expressing tumours had significantly reduced p53 staining, indicating that the specific shRNAs are effective in silencing the expression of the respective mutant p53 *in vivo* during tumour growth (Fig. 7B). This data establishes that mutant p53-specific siRNAs are effective in retarding tumour cell growth *in vivo*.

[0092] In addition, it was examined if the growth of R249S mutant expressing, patient-derived triple-negative breast cancer xenograft tumours (PDX) could be influenced by the siRNAs utilized in a therapeutic treatment protocol. In essence, PDX tumours were grown orthotopically, and when they reached 170mm³, mice were treated twice weekly with scrambled siRNA or mutant p53-specific siRNA that was delivered intravenously in nano-liposomes, which have been shown to effectively deliver to tumours. Treatment with si-6 (against R249S) twice weekly led to growth retardation of the tumours when compared to

scrambled siRNA treated mice, which developed to full blown tumours by 29 days post-treatment (data not shown). Immunohistochemical staining for p53 indicated that the expression of mutant p53 was significantly reduced in the si-6 treated tumours (data not shown). Further analysis of multiple organs at sacrifice from siRNA treated mice did not show any abnormalities, excluding any side effects due to this treatment regimen (data not shown). Taken together, this data establishes that mutant p53-specific siRNAs can be used therapeutically to retard tumour growth *in vivo*.

TABLES

Table 1: Human tumour cell lines used

CELL LINES USED IN STUDY	P53 STATUS	ORIGIN OF CELL LINE
A549	WILD-TYPE	LUNG CARCINOMA
A375	WILD-TYPE	MALIGNANT MELANOMA
HCT116	WILD-TYPE	COLON CARCINOMA
RKO	WILD-TYPE	COLON CARCINOMA
AU565	R175H	BREAST CARCINOMA
SKBR3	R175H	BREAST CARCINOMA
HCC1395	R175H	BREAST CARCINOMA
HEC-1A	R248Q	ENDOMETRIAL ADENOCARCINOMA
RD	R248W	RHABDOMYOSARCOMA
786-O	R248W	KIDNEY ADENOCARCINOMA
COLO-320DM	R248W	COLON ADENOCARCINOMA
PLC-PRF/5	R249S	HEPETOCELLULAR CARCINOMA
KNS-62	R249S	BRONCHIAL SQUAMOUS CANCER
BT549	R249S	BREAST CARCINOMA
ASPC1	R273H	PANCREATIC ADENOCARCINOMA
WIDR1	R273H	COLON ADENOCARCINOMA
H1975	R273H	LUNG CARCINOMA

SEQUENCES

[0093] A wildtype p53 polypeptide may comprise or consist of the amino acid sequence of UniProtKB – P04637 (P53_HUMAN):

MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPDDIEQWFTEDPGPDEA
 PRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSSVPSQKTYQGSYGFR LGFLHSGTAKSVTCTY
 SPALNKMFCQLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHHERCSDSDG
 LAPPQHILIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYMCNSSCMGGMNR
 RPILTIITLEDSSGNLLGRNSFEVRVCACPGRRRTEENLRKKGEPHHELPPGSTKRALPNNT
 SSSPQPKKKPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKG
 QSTRHKKLMFKTEGPDSD (SEQ ID NO: 1)

SEQ ID NO:	Mutation	Sequence (mutated residue relative to wildtype p53 shown bold, underlined)
2	R175H	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTEVVR <u>H</u> CPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYCNS SCMGGMNR RPILTIITLEDSSGNLLGRNSFEVRVCACPGRRRTEENLR RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTRHKKLMFK TEGPDSD
3	R248W	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYCNS SCMGGMN <u>W</u> RPILTIITLEDSSGNLLGRNSFEVRVCACPGRRRTEENLR RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTRHKKLMFK TEGPDSD
4	R273H	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFR LGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYCNS SCMGGMNR RPILTIITLEDSSGNLLGRNSFEV <u>H</u> VCACPGRRRTEENLR RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTRHKKLMFK

SEQ ID NO:	Mutation	Sequence (mutated residue relative to wildtype p53 shown bold, underlined)
		TEGPDS
5	R249S	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPGPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFRGLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTVEVRRCPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYCNS SCMGGMNRS <u>P</u> ILTIITLEDSSGNLLGRNSFEVRVCACPGRRRTEENL RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFK TEGPDS
36	R249M	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPGPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFRGLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTVEVRRCPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYCNS SCMGGMNRM <u>P</u> ILTIITLEDSSGNLLGRNSFEVRVCACPGRRRTEENL RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFK TEGPDS
37	R249G	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPGPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFRGLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTVEVRRCPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYCNS SCMGGMNRR <u>G</u> PILTIITLEDSSGNLLGRNSFEVRVCACPGRRRTEENL RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFK TEGPDS
38	R273L	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPGPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFRGLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTVEVRRCPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNMYCNS SCMGGMNRRPILTIITLEDSSGNLLGRNSFEV <u>L</u> VCACPGRRRTEENL

SEQ ID NO:	Mutation	Sequence (mutated residue relative to wildtype p53 shown bold, underlined)
		RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFK TEGPDSD
39	R248Q	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPD DIEQWFTEDPGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFRGLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQ LWVDSTPPPGTRVRAMAIYKQSQHMTVEVRRCPHHERCSDSDGLAPP QHLIRVEGNLRVEYLDDRNTFRHSVVPYEPPEVGSDDCTTIHYNMCNS SCMGGMN <u>Q</u> RPILTIITLEDSSGNLLGRNSFEVRVCACPRDRRTEENL RKKGEPHHELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERF EMFRELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFK TEGPDSD

SEQ ID NO:	Sequence	Name/comment
6	5' AUG ACG GAG GUU GUG AGG CgC UGC CCC CAC CAU GAG 3'	R175H P53 WT
7	5' AUG ACG GAG GUU GUG AGG CaC UGC CCC CAC CAU GAG 3'	R175H P53 MUT-R175H
8	5' UG ACG GAG GUU GUG AGG CaC U 3'	R175H Si-1-R175H
9	5' AGG CaC UGC CCC CAC CAU GA 3'	R175H Si-2-R175H
10	5' UGC AUG GGC GGC AUG AAC cGG AGG CCC AUC CUC 3'	R248W P53 WT
11	5' UGC AUG GGC GGC AUG AAC uGG AGG CCC AUC CUC 3'	R248W P53 MUT-R248W
44	5' UGC AUG GGC GGC AUG AAC CaG AGG CCC AUC CUC 3'	R248Q P53 MUT-R248Q
12	5' GC AUG AAC uGG AGG CCC AU 3'	R248W/Q Si-3-R248W/R248Q
13	5' GC GGC AUG AAC uGG AGG CCC 3'	R248W/Q Si-4-R248W/R248Q
14	5' UGC AUG GGC GGC AUG AAC CGG AGg CCC AUC CUC ACC 3'	R249S P53 WT
15	5' UGC AUG GGC GGC AUG AAC CGG AGu CCC AUC CUC ACC 3'	R249S P53 MUT-R249S
45	5' UGC AUG GGC GGC AUG AAC CGG AuG CCC AUC CUC ACC 3'	R249M P53 MUT-R249M
46	5' UGC AUG GGC GGC AUG AAC CGG gGG CCC AUC CUC ACC 3'	R249G P53 MUT-R249G
16	5' G AAC CGG AGu CCC AUC CUC 3'	R249S/M/G Si-5-R249S/ R249M/R249G

SEQ ID NO:	Sequence	Name/comment
17	5' AAC CGG AGu CCC AUC CUC A 3'	R249S/M/G Si-6-R249S/R249M/R249G
18	5' AGC UUU GAG GUG CgU GUU UGU GCC UGU CCU 3'	R273H P53 WT
19	5' AGC UUU GAG GUG CaU GUU UGU GCC UGU CCU 3'	R273H P53 MUT-R273H
47	5' AGC UUU GAG GUG CuU GUU UGU GCC UGU CCU 3'	R273L P53 MUT-R273L
20	5' AG GUG CaU GUU UGU GCC UGU 3'	R273H/L Si-7-R273H/R273L
21	5' GAG GUG CaU GUU UGU GCC UGU 3'	R273H/L Si-8-R273H/R273L
22	5' CAC UGC CCC CAC CAU GAG CG 3'	General p53; si-p53
23	5' UUC UCC GAA CGT GUC ACG U 3'	General scrambled controls
24; 40; 41	5'-AAGCTTTN ₍₁₉₋₂₉₎ (sense sequence)TTCAAGAGAN ₍₁₉₋₂₉₎ (antisense sequence)TTTTTTA-3'	Exemplary shRNA upper oligonucleotide, whereby (other than the siRNA sequence which is referred to as N ₍₁₉₋₂₉₎) the nucleotides indicated at the front and end of each oligonucleotide are for the restriction enzyme cutting site. The middle sequence in each case is for the formation of stem loop.
25; 42; 43	5'-AGCTTAAAAAN ₍₁₉₋₂₉₎ (sense sequence)TCTCTTGAAN ₍₁₉₋₂₉₎ (antisense sequence)GGG-3'	Exemplary shRNA lower oligonucleotide, whereby (other than the siRNA sequence which is referred to as N ₍₁₉₋₂₉₎) the nucleotides indicated at the front and end of each oligonucleotide are for the restriction enzyme cutting site. The middle sequence in each case is for the formation of stem loop.
26	Forward 5'- AAGCTTAGGCaCTGCCCCCACCATGAT TCAAGAGATCATGCTGGGGGCAGGCC TTTTTA-3'	shRNA vs R175H (sh-2) forward
27	Reverse 3'- GAATCCGTGACGGGGGTGGTACTAAG TTCTCTAGTACCACCCCGTCACGGAA AAATTCGA -5'	shRNA vs R175H (sh-2) reverse
28	Forward 5'- AAGCTTGCGGCATGAAAGGGAGGCCCT TCAAGAGAGGGCCTCCAGTTCATGCCG	shRNA vs R248W/R248Q (sh-4) forward

SEQ ID NO:	Sequence	Name/comment
	<u>CTTTT</u> TA -3'	
29	Reverse 3'- GAACGCGCGTACTTGACCTCCGGGAAGT TCTCTCCCGGAGGTCAAGTACGGCGAA AAATTCGA -5'	shRNA vs R248W/R248Q (sh-4) reverse
30	Forward 5'- AAGCTTAACCGGAGGCCCATCCTCATTC AAGAGATGAGGATGGGACTCCGGTITT TTA -3'	shRNA vs R249S/R249M/R249G (sh-6) forward
31	Reverse 3'- GAATTGGCCTCAGGGTAGGAGTAAGTT CTCTACTCCTACCCTGAGGCCAAAAA TTCGA -5'	shRNA vs R249S/R249M/R249G (sh-6) reverse
32	Forward 5'- AAGCTTGAGGTGCATGTTTGTGCCTGTT CAAGAGAACAGGCACAAACAIGCACCT CTTTTA -3'	shRNA vs R273H/ R273L (sh-8) forward
33	Reverse 3'- GAACTCCACGTACAAACACGGACAAA GTTCTCTTGTCCGTGTTTGTACGTGGA GAAAATTCGA -5'	shRNA vs R273H/ R273L (sh-8) reverse
34	Forward 5'- AAGCTTTTCTCCGAACGTGTCACGTTT CAAGAGAACGTGACACGTTCGGAGAA TTTTTA -3'	shRNA vs scramble (sh-scr) forward
35	Reverse 3'- GAAAAGAGGCTTGCACAGTGCAAAGT TCTCTTGCACGTGTGCAAGCCCTCTTAAA AATTCGA -5'	shRNA vs scramble (sh-scr) reverse

CLAIMS

1. A nucleic acid sequence for targeting a single point mutation within the p53 tumour suppressor gene wherein:
 - (a) the single point mutation is selected from R249S (p53), R249G (p53) and R249M (p53) and the nucleic acid sequence is an siRNA sequence selected from SEQ ID NOs 16 and 17 or an shRNA sequence having an siRNA sequence selected from SEQ ID NOs 16 and 17;
 - (b) the single point mutation is selected from R248W (p53) and R248Q (p53) and the nucleic acid sequence is an siRNA sequence which is SEQ ID NO: 13 or an shRNA sequence having an siRNA sequence which is SEQ ID NO: 13; or
 - (c) the single point mutation is selected from R273H (p53) and R273L (p53) and the nucleic acid sequence is an siRNA sequence which is SEQ ID NO: 21 or an shRNA sequence having an siRNA sequence which is SEQ ID NO: 21.
2. The nucleic acid sequence of claim 1, wherein the nucleic acid sequence:
 - (a) results in any one or more of the following effects selected from the group consisting of cell death, abrogation of addiction, activation of any one or more p53 target genes, relief of a dominant negative effect, increased sensitivity to one or more anti-cancer agents, and retardation or halting of tumour growth; and/or
 - (b) is capable of substantially silencing mutant p53 tumour suppressor gene alleles.
3. The nucleic acid sequence of any one of the preceding claims, wherein the shRNA sequence comprises stems with the length of between 15 to 30 base pairs.
4. A nucleic acid sequence which is:
 - (a) an siRNA sequence selected from SEQ ID NOs 17, 13, 16 and 21; or
 - (b) an shRNA sequence having an siRNA sequence selected from SEQ ID NOs 17, 13, 16 and 21.
5. A nucleic acid sequence which encodes an shRNA sequence according to any one of the preceding claims.
6. The nucleic acid sequence of claim 5 comprising:
 - (a) SEQ ID NOs 24, 40 and 41;

- (b) SEQ ID NOs 25, 42 and 43; or
 - (c) SEQ ID NO: 28, 29, 30, 31, 32 or 33.
7. A double stranded siRNA molecule in which the sense strand consists of the sequence in SEQ ID NO 17, 13, 16 or 21.
 8. A nucleic acid sequence according to any one of claims 1 to 5 or a double stranded siRNA molecule according to claim 7 for use in therapy.
 9. One or more nucleic acid sequence according to any one of claims 1 to 5 or one or more double stranded siRNA molecule according to claim 7 for use in a method of treating cancer in a subject having one or more single point mutations within the p53 tumour suppressor gene at one more sites, wherein:
 - (a) the subject has a single point mutation in the p53 tumour suppressor gene at R249(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence selected from SEQ ID NOs 16 and 17, an shRNA sequence having an siRNA sequence selected from SEQ ID NOs 16 and 17 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 16 or 17; and/or
 - (b) the subject has a single point mutation in the p53 tumour suppressor gene at R248(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence which is SEQ ID NO: 13, an shRNA sequence having an siRNA sequence which is SEQ ID NO: 13 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 13; and/or
 - (c) the subject has a single point mutation in the p53 tumour suppressor gene at R273(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence which is SEQ ID NO: 21, an shRNA sequence having an siRNA sequence which is SEQ ID NO: 21 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 21.
 10. One or more nucleic acid sequence or one or more double stranded siRNA molecule for use according to claim 9, wherein the cancer is:

- (a) selected from the group consisting of oesophagus, upper respiratory tract, skin, epithelial, central nervous system, ovarian, breast, gastro-intestinal, large intestines, small intestines, colorectal, liver, adenocarcinoma, adrenal adenocarcinoma, thyroid, lung, pancreas, kidney, endometrial, hematopoietic, muscles, connective tissue (such as tendon or cartilage), bone, soft tissue, lymphoid tissue, lymph and the immune system;
 - (b) selected from the group consisting of melanomas, myelomas, carcinomas, sarcomas, lymphomas, blastomas and germ cell tumours;
 - (c) selected from the group consisting of lung carcinoma, malignant melanoma, colon carcinoma, breast carcinoma, endometrial adenocarcinoma, rhabdomyosarcoma, kidney adenocarcinoma, colon adenocarcinoma, hepatocellular carcinoma, bronchial squamous cancer, ovarian carcinoma and pancreatic adenocarcinoma; and/or
 - (d) dependent on the p53 tumour suppressor gene.
11. One or more nucleic acid sequence or one or more double stranded siRNA molecule for use according to claim 9 or 10, wherein the one or more point mutations are selected from the group consisting of R249S (p53), R249G (p53), R249M (p53), R248W (p53), R248Q (p53), R273H (p53) and R273L (p53), and combinations thereof.
 12. One or more nucleic acid sequence or one or more double stranded siRNA molecule for use according to any one of claims 9 to 11, wherein administration of the one or more of the nucleic acid sequences or one or more double stranded siRNA molecules results in one or more of the following effects selected from the group consisting of cell death, abrogation of addiction, activation of any one or more p53 target genes, relief of a dominant negative effect, increased sensitivity to one or more anti-cancer agents, and retardation or halting of tumour growth.
 13. One or more nucleic acid sequence or one or more double stranded siRNA molecule for use according to any one of claims 9 to 12, wherein the nucleic acid sequence is for use with a therapeutic agent.
 14. One or more nucleic acid sequence or one or more double stranded siRNA molecule for use according to claim 13, wherein the therapeutic agent is an anti-cancer agent.

15. Use of one or more nucleic acid sequence according to any one of claims 1 to 5 or one or more double stranded siRNA molecule according to claim 7 for the manufacture of a medicament for treating cancer in a subject having one or more single point mutations within the p53 tumour suppressor gene at one or more sites, wherein:
 - (a) the subject has a single point mutation in the p53 tumour suppressor gene at R249(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence selected from SEQ ID NOs 16 and 17, an shRNA sequence having an siRNA sequence selected from SEQ ID NOs 16 and 17 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 16 or 17; and/or
 - (b) the subject has a single point mutation in the p53 tumour suppressor gene at R248(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence which is SEQ ID NO: 13, an shRNA sequence having an siRNA sequence which is SEQ ID NO: 13 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 13; and/or
 - (c) the subject has a single point mutation in the p53 tumour suppressor gene at R273(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence which is SEQ ID NO: 21, an shRNA sequence having an siRNA sequence which is SEQ ID NO: 21 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 21.

16. Use of one or more nucleic acid sequence or one or more double stranded siRNA molecule for the manufacture of a medicament according to claim 15 wherein the cancer is:
 - (a) selected from the group consisting of oesophagus, upper respiratory tract, skin, epithelial, central nervous system, ovarian, breast, gastro-intestinal, large intestines, small intestines, colorectal, liver, adenocarcinoma, adrenal adenocarcinoma, thyroid, lung, pancreas, kidney, endometrial, hematopoietic, muscles, connective tissue (such as tendon or cartilage), bone, soft tissue, lymphoid tissue, lymph and the immune system;
 - (b) selected from the group consisting of melanomas, myelomas, carcinomas, sarcomas, lymphomas, blastomas and germ cell tumours;
 - (c) selected from the group consisting of lung carcinoma, malignant melanoma, colon carcinoma, breast carcinoma, endometrial adenocarcinoma, rhabdomyosarcoma, kidney adenocarcinoma, colon adenocarcinoma, hepatocellular carcinoma, bronchial squamous cancer, ovarian carcinoma and pancreatic adenocarcinoma; and/or

(d) dependent on the p53 tumour suppressor gene.

17. Use of one or more nucleic acid sequence or one or more double stranded siRNA molecule for the manufacture of a medicament according to claim 15 or 16 wherein the one or more point mutations are selected from the group consisting of R249S (p53), R249G (p53), R249M (p53), R248W (p53), R248Q (p53), R273H (p53) and R273L (p53), and combinations thereof.
18. Use of one or more nucleic acid sequence or one or more double stranded siRNA molecule for the manufacture of a medicament according to any one of claims 15 to 17 wherein administration of the one or more of the nucleic acid sequences or one or more double stranded siRNA molecules results in one or more of the following effects selected from the group consisting of cell death, abrogation of addiction, activation of any one or more p53 target genes, relief of a dominant negative effect, increased sensitivity to one or more anti-cancer agents, and retardation or halting of tumour growth.
19. Use of one or more nucleic acid sequence or one or more double stranded siRNA molecule for the manufacture of a medicament according to any one of claims 15 to 18 wherein the medicament is to be administered with a therapeutic agent, optionally wherein the therapeutic agent is an anti-cancer agent.
20. Use of one or more nucleic acid sequence according to any of claims 1 to 5 or one or more double stranded siRNA molecule according to claim 7 for the manufacture of a medicament for treating a subject susceptible to treatment, said susceptible subject being identified as having one or more single point mutations within the p53 tumour suppressor gene at one or more sites, wherein:
 - (a) the subject is identified as having a single point mutation in the p53 tumour suppressor gene at R249(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence selected from SEQ ID NOs 16 and 17, an shRNA sequence having an siRNA sequence selected from SEQ ID NOs 16 and 17 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 16 or 17; and/or
 - (b) the subject is identified as having a single point mutation in the p53 tumour suppressor gene at R248(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence which is SEQ ID NO: 13, an shRNA sequence having an siRNA sequence which is SEQ ID NO: 13

and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 13; and/or

(c) the subject is identified as having a single point mutation in the p53 tumour suppressor gene at R273(p53) and the one or more nucleic acid sequence or one or more double stranded siRNA molecule is selected from an siRNA sequence which is SEQ ID NO: 21, an shRNA sequence having an siRNA sequence which is SEQ ID NO: 21 and a double stranded siRNA molecule in which the sense sequence consists of the sequence in SEQ ID NO 21.

21. Use of one or more nucleic acid sequence or one or more double stranded siRNA molecule for the manufacture of a medicament according to claim 20 wherein the one or more point mutations are selected from the group consisting of R249S (p53), R249G (p53), R249M (p53), R248W (p53), R248Q (p53), R273H (p53) and R273L.