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(54) Title: SELECTIVE KILLING OF CANCER CELLS

Fig. 1

(57) Abstract: There is provided a method for eliminating a tumor cell with a hyperactivated B-Raf or hyperactivated Ras kinase and increased DUSP6 activity. Specifically, the invention provides a method of contacting a tumor cell with a hyperactivated B-Raf kinase or a hyperactivated Ras kinase with an agent that reduces DUSP6 activity. Also, there is provided a diagnostic test for therapy responder prediction.
Selective Killing of Cancer Cells

FIELD OF THE INVENTION

The present invention is directed to the targeting of a tumor cell with a hyperactivated BRAF kinase and/or with hyperactivated RAS kinases and increased DUSP6 activity. Specifically, the invention provides a method of contacting a tumor cell with a hyperactivated BRAF kinase and/or with hyperactivated RAS kinases with an agent that reduces DUSP6 activity. Also, the invention provides a diagnostic test for therapy responder prediction.

BACKGROUND OF THE INVENTION

One approach to the study of cancer is profiling of gene activities, an effort aimed at identifying perturbations in gene expression that lead to the malignant phenotype. However, cancers differ widely in their gene activity "signatures", and the signatures are often not directly informative, leading to difficulties in diagnosis and treatment, as well as in the development of effective therapeutics. The major reason for these difficulties is that the function of a gene is often unknown and that it can differ depending on the presence or absence of other molecular changes in the tumor cells.

Determining the functions of genes in tumors and how this function is influenced by other molecular changes may provide a more effective approach for future cancer management and/or treatment. Accordingly, there is a need in the art to better understand the functions of genes depending on different mutation or activity profiles of specific classes of tumors to provide improved therapeutics, diagnostics and screening methods.

The Raf/Ras protein serine/threonine kinase family consists of six members, A-Raf, B-Raf, C-Raf, H-Ras, K-Ras, and N-Ras. Raf/Ras family members are signaling molecules in the MAPK (Ras/Raf/MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK)) pathway, which is a signal transduction pathway that relays extracellular signals from the cell membrane to the nucleus via an ordered series of consecutive phosphorylation events. Typically, an extracellular ligand binds to its tyrosine kinase receptor, leading to Ras kinase activation and initiation of a cascade of phosphorylation events. Activated Ras kinases cause phosphorylation and activation of Raf kinases, which in turn phosphorylate and
activate MEK1/2. MEK kinases in turn phosphorylate and activate ERK1/2, which phosphorylates several cytoplasmic and nuclear targets that ultimately lead to expression of proteins playing important roles in cell growth and survival.

A hyperactivation of Raf/Ras family members is well known to play a role in diverse cancer types, and results in increased activity of downstream components of the Raf/Ras-MEK-ERK signalling pathway, cumulating in increased phosphorylation of ERK1/2. Events that lead to Raf/Ras hyperactivation are well known to the art and can, for example, include hyperactivating mutations, gene amplifications, increased mRNA expression levels or increased protein expression levels.

Hyperactivation of B-Raf and Ras kinases occurs over a broad spectrum of cancer types.

The highest frequency of B-Raf hyperactivation is found in malignant melanoma (27%-70%), papillary thyroid cancer (36%-53%), colorectal cancer (5%-22%) and serous ovarian cancer (30%), but it also occurs at a low frequency (1%-3%) in a wide variety of other cancers. Many different mutations in BRAF have been identified, but most mutations are extremely rare. The V600E mutation predominates, representing 86% of BRAF mutations in cancer (reviewed by Garnett and Marais 2004).

A systematic scan of 545 cancer cell lines revealed that 71 (13%) had hyperactivating mutations of RAS genes: 14 of 40 (35%) colorectal cancers, 23 of 131 (18%) lung cancers (22 non-small cell lung cancer NSCLC, 1 small-cell lung cancer SCLC), 3 of 3 (100%) pancreatic cancers, 3 of 34 (9%) melanomas, 4 of 26 (15%) ovarian cancers, 3 of 27 (11%) neuroblastomas, 2 of 10 (20%) bladder cancers, 9 of 53 (17%) leukemias/lymphomas (Davies et al., 2002). Hyperactivation of the family member N-Ras by mutation is most frequently found in thyroid cancer (28-50%; Fukushima et al., 2003), melanoma (20%) and liver cancer (20%), followed by leukemia (18%), soft tissue (17%), urinary tract (17%), and ovarian cancer (9%), but also occurs at lower frequency (1-9%) in various other cancer types (http://www.sanger.ac.uk/perl/genetics/). In the NRAS gene the 182A>G [Q61R] mutation is the most frequently found hyperactivating mutation, but other mechanisms for hyperactivation of Ras family members in general and N-Ras in specific are known to the art.
Mutations that lead to hyperactivation of B-Raf have been found in 50-60% of the sporadic melanomas, making BRAF the most often mutated gene in melanomas that is known up to date. The majority of BRAF mutations occur as a result of a single base missense substitution that converts T to A at nucleotide 1799 which substitutes a Valine for a Glutamic Acid at codon 600 (V600E) in exon 15. The mutation was originally misidentified as occurring at codon 599 instead of codon 600, therefore it is referred to as either V600E or V599E in the literature (Sharma et al. 2006).

This mutation increases basal kinase activity of B-Raf, resulting in hyperactivity of the MAPK pathway evidenced by constitutively elevated activity of downstream kinases MEK and ERK. B-RAF mutations are acquired. Somatic, post-zygotic events have not been identified in familial melanomas.

Of the Ras proteins, N-Ras is frequently hyperactivated in about 20% of the malignant melanoma. In the majority of the cases, this is caused by the Q61R mutation, resulting in hyperactivity of the MAPK pathway, as evidenced by constitutively elevated activity of downstream kinases MEK and ERK.

Of the three major forms of skin cancer, malignant melanoma carries the highest risk of mortality from metastasis. The prognosis for patients in the late stages of this disease remains very poor with average survival from six to ten months.

Currently, there is no effective long-term treatment for patients suffering from the advanced stages of this cancer despite many clinical trials testing the efficacy of a wide variety of therapeutics ranging from surgery to immune-, radio- and chemotherapy. The lack of effective therapeutic regimes is due, in part, to a lack of more comprehensive information about the genes altered during melanoma development, their functions in the background of different changes, such as B-Raf and N-Ras hyperactivation, and therapies specifically targeted to correct these defects.

Patients with metastatic (Stage IV) malignant melanoma have a median survival of approximately one year. Current standard treatment consists of combination chemotherapy with agents such as cisplatin, DTIC, and BCNU, with or without cytokines such as interleukin-2 (IL-2) or interferon-a (IFN-a). Response rates to chemotherapy have been reported to be as high as 60%, yet only approximately 5% of patients experience long-term
survival, regardless of the therapeutic regimen employed. Conventional chemotherapy aims to control the growth of cancer by targeting rapidly growing cells.

However, this effect is not specific, as many normal cells, such as those of the bone marrow and the intestinal epithelium, also have a basal level of proliferation. Therefore, many normal cells of the body also are susceptible to the toxic effects of chemotherapy, and conventional chemotherapy can impart a substantial degree of morbidity to the patient. The toxic effects limit the dose that is applicable to the patient, which in turn results in inefficient elimination of the cancer cells, so that either no response, rapid fading of the response and/or the development of resistance is observed.

Clearly, new approaches to the treatment of metastatic melanoma are needed.

B-Raf-inhibitors such as the drug PLX4032 are presently under investigation in clinical trials. While the initial data point to good response rates in melanoma patients with hyperactivated B-Raf kinase (Flaherty et al., 2010), there are also first hints that the tumor cells may develop resistance against such drugs via hyperactivation of N-Ras (Nazarian et al., 2010; Johannessen et al., 2010), which defines a still persisting demand for novel targeted therapeutic approaches.

RNA interference (RNAi) is a polynucleotide sequence-specific, post transcriptional gene silencing mechanism elicited by double-stranded formation of a small RNA-oligonucleotide with the messenger RNA (mRNA) of a target gene that results in degradation or in inhibition of translation of a specific mRNA, thereby reducing the expression of a desired target polypeptide encoded by the mRNA. RNAi is mediated by single-stranded RNA-polynucleotides that are commonly administered to the cells as double-stranded molecules as also described herein below, for example, double-stranded RNA (dsRNA), having sequences that correspond to exonic sequences encoding portions of the polypeptides for which expression is to be compromised or dsRNAs that correspond to the 5′- or 3′- untranslated regions (5′-UTRs; 3′-UTRs) of the mRNA that shall be inactivated.

siRNA polynucleotides may offer certain advantages over other polynucleotides known to the art for use in sequence-specific alteration or modulation of gene expression to yield altered levels of an encoded polypeptide product. These advantages include lower effective siRNA polynucleotide concentrations, enhanced siRNA polynucleotide stability, and shorter
siRNA polynucleotide oligonucleotide lengths relative to other polynucleotides (e.g., antisense, ribozyme or triplex polynucleotides). By way of a brief background, "antisense" polynucleotides bind in a sequence-specific manner to target nucleic acids, such as mRNA or DNA, to prevent transcription of DNA or translation of the mRNA (see, e.g., U.S. Pat. No. 5,168,053). "Ribozyme" polynucleotides can be targeted to any RNA transcript and are capable of catalytically cleaving such transcripts, thus impairing translation of mRNA (see, e.g., U.S. Pat. No. 5,272,262). "Triplex" DNA molecules refer to single DNA strands that bind duplex DNA to form a collinear triplex molecule, thereby preventing transcription (see, e.g., U.S. Pat. No. 5,176,996, describing methods for making synthetic oligonucleotides that bind to target sites on duplex DNA). Such triple-stranded structures are unstable and form only transiently under physiological conditions. Because single stranded polynucleotides do not readily diffuse into cells and are therefore susceptible to nuclease digestion, development of single-stranded DNA for antisense or triplex technologies often requires chemically modified nucleotides to improve stability and absorption by cells. siRNAs, by contrast, are readily taken up by intact cells, are effective at interfering with the expression of specific polypeptides at concentrations that are several orders of magnitude lower than those required for either antisense or ribozyme polynucleotides, and do not require the use of chemically modified nucleotides.

As mentioned above malignant melanoma is the skin cancer with the most significant impact on man carrying the highest risk of death from metastases. Both incidence and mortality rates continue to rise each year, with no effective long-term treatment on the horizon. In part, this reflects the lack of knowledge about critical genes involved in melanoma and specific therapies targeted to correct these defects. Accordingly, a need exists in the art for identification of critical genes involved in melanoma and specific therapies targeted to correct these defects, for example by a targeted reduction of the activity of key genes/effectors in the relevant signalling pathways.

Identifying a gene as a selective target provides new therapeutic opportunities for cancer patients. Simultaneously, it provides methods for the identification of a patient that would profit from such therapy in the form of diagnostic kits. A distinction between patients that profit and patients that do not profit from a given treatment strategy is of critical importance for the treatment success and for avoiding unnecessary treatment of patients.
US 2008/0131885 A1 discloses a method for selecting an individual for treatment with a Ras/Raf/MEK/ERK pathway inhibitor, comprising: (a) obtaining a cancerous tumor sample from said individual; (b) measuring the amount of a biomarker in said tumor sample, wherein said biomarker can be DUSP6; and (c) selecting said individual for treatment with a Ras/Raf/MEK/ERK pathway inhibitor based on the measured amount of said biomarker. The cancerous tumor can be a skin tumor, e.g. a melanoma and said tumor can comprise a B-Raf mutation.

WO 2009/140409 A1 discloses a method for predicting a likelihood that a human patient having a K-Ras-negative EGFR-expressing cancer will exhibit a beneficial response to an EGFR inhibitor comprising: (a) measuring, in a tumor sample obtained from the patient, an expression level of at least one response indicator gene, or its expression product, e.g. DUSP6; (b) normalizing the expression level to obtain a normalized expression level; (c) using the normalized expression level to determine the likelihood that the patient will exhibit a beneficial response to the EGFR inhibitor; and (d) generating a report based on the determined likelihood. WO 2009/140409 A1 further discloses an array comprising a plurality of polynucleotides immobilized on a surface of a solid support suitable for carrying out the method and further comprising a probe that hybridizes under stringent hybridization conditions to a nucleic acid comprising an activating K-Ras mutation.

Therefore, it is a primary object of the present invention to improve the state of the art methods for diagnosing and treating cancer, including malignant melanoma.

It is a further object of the present invention to provide a method for reducing DUSP6 activity in a cancer cell, and thereby achieving a positive therapeutic effect, for example by induction of apoptosis or inhibition of cancer growth.

It is a further object of the present invention to provide a method for achieving a positive therapeutic effect by inducing apoptosis in or a growth reduction of a cancer cell with an agent that reduces DUSP6 activity.

It is a further object of the present invention to provide a combinatorial approach of treating cancer by inducing apoptosis in or conferring growth reduction to a cancer cell, in combination with other therapeutic strategies, which, for example induce apoptosis,
decrease cell proliferation or growth of the tumor cell, stimulate an anti-tumor immune response, and/or inhibit vascularization of the tumor.

It is a further object of the present invention to provide a method of treating cancer, including melanomas, that reduces tumor size more efficiently than conventional methods.

It is a further object of the present invention to provide a method of treating cancer, including melanomas, that requires a lower concentration of chemotherapy to be used, thereby decreasing toxicity to the patient.

It is a further object of the present invention to provide a method of treating cancer, including melanomas, that aims at decreasing the emergence of resistance against B-Raf inhibitors.

It is a further object of the present invention to provide a method for diagnosing, which cancer patients may profit from such therapies.

These and other objects, features, or advantages will become evident from the following description of the invention.

The prior art does not disclose or indicate to use an agent that reduces DUSP6 activity for treating cancer. The present inventors have unexpectedly found that DUSP6 inhibition confers anticancer properties. This is surprising since up-regulated DUSP6 is understood as a natural defence towards cancer. DUSP6 is a tumor suppressor, wherefore it is very surprising that reducing its activity confers anti-cancer activity. The present inventors have found that reducing DUSP6 activity indeed confers cancer cell death by inducing apoptosis in cancer cells.

SUMMARY OF THE INVENTION

The present invention is based on the present inventors' discovery that inhibiting DUSP6 selectively confers cell death and/or growth reduction to cancer cells with DUSP6 hyperactivation in combination with either B-Raf or Ras hyperactivation.
In one embodiment, the invention provides a method for killing a tumor cell having a mutation or hyperactivity of B-Raf and/or Ras kinases and preferably increased DUSP6 activity by reducing DUSP6 activity.

In another embodiment, the invention provides a method for killing a tumor cell having a mutation or hyperactivity of B-Raf and/or Ras kinases and preferably increased DUSP6 activity, comprising contacting a tumor cell with an agent that reduces DUSP6 activity.

In yet another embodiment, the tumor cell is a melanoma cell.

In a further embodiment this provides a diagnostic kit for identifying a patient that would profit from such therapy, comprising a detection method for increased DUSP6 levels alone or in combination with detection of mutation or hyperactivity of B-Raf and/or Ras kinases.

The present inventors contemplate a method for destroying a tumor with a B-Raf and/or Ras kinase hyperactivation and preferably with increased DUSP6 activity in a patient, comprising the steps of administering to a cancer patient an effective amount of an agent to inactivate DUSP6, and optionally administering additionally to the cancer patient an effective amount of another therapeutic agent to achieve a positive therapeutic effect.

Also disclosed herein is a method for destroying cancer cells with B-Raf and/or Raf kinase hyperactivation and preferably with increased DUSP6 activity in a patient comprising the steps of: administering to a tumor in a mammal an effective amount of an agent that reduces DUSP6 activity in conjunction with administering to the tumor an effective amount of an agent that reduces B-Raf activity, thereby treating the tumor.

Also disclosed herein is a method for destroying cancer cells with B-Raf hyperactivation that has become resistant to B-Raf inhibitors via N-Ras hyperactivation.

Also disclosed herein is a method for avoiding resistance against B-Raf inhibitors of cancer cells with B-Raf hyperactivation by administering to a tumor in a mammal an effective amount of an agent that reduces DUSP6 activity in conjunction with administering to the tumor an effective amount of an agent that reduces B-Raf activity, thereby treating the tumor.
Also disclosed herein is a method for avoiding resistance against Ras inhibitors of cancer cells with Ras hyperactivation by administering to a tumor in a mammal an effective amount of an agent that reduces DUSP6 activity in conjunction with administering to the tumor an effective amount of an agent that reduces Ras activity, thereby treating the tumor.

Also disclosed herein is a method for avoiding resistance against N-Ras inhibitors of cancer cells with N-Ras hyperactivation by administering to a tumor in a mammal an effective amount of an agent that reduces DUSP6 activity in conjunction with administering to the tumor an effective amount of an agent that reduces N-Ras activity, thereby treating the tumor.

Preferably the methods of the present invention involves contacting of said cancer cells with a DUSP6 inhibiting agent, where the agent is a siRNA molecule, an antisense molecule, an antagonist, a ribozyme, an inhibitor, a small molecules or a peptide, that acts as a pseudosubstrate for DUSP6, such as a pseudosubstrate for a catalytic domain or a regulatory domain of DUSP6. Preferably the agent is an agent that acts as a competitive inhibitor for DUSP6, such as a competitive inhibitor for a catalytic domain of DUSP6 or an agent that results in downregulation of DUSP6 protein levels. The agent may be a siRNA selected from Table 1 or Table 2. In the most preferred embodiment of the present invention the agent is a siRNA molecule that comprises a polynucleotide selected from the group having a sequence of GUGCAACAGACUCGGAGGUA, AGCUCAACUGUCGAUGGAA, and the complements thereof.

Preferably the methods of the present invention involves contacting of said cancer cells using one of more the following vehicles: a liposome, a nanoliposome, a ceramide-containing nanoliposome, a proteoliposome, a nanoparticulate, a calcium phosphor-silicate nanoparticulate, a calcium phosphate nanoparticulate, a silicon dioxide nanoparticulate, a nanocrystalline particulate, a semiconductor nanoparticulate, poly(D-arginine), a nanodendrimer, a virus, calcium phosphate nucleotide-mediated nucleotide delivery, electroporation, and microinjection.

In another aspect, the invention provides a pharmaceutical composition for eliminating cancer cells comprising: an agent that reduces DUSP6 activity and a carrier.
In certain embodiments of this method, the sample is a sample of melanoma tissue. In certain other embodiments of this method, the sample is selected from the group consisting of cancers with hyperactivated B-Raf kinase (eg. by the V600E mutation) and/or hyperactivated Ras kinases (eg. by the Q61R mutation in N-Ras) and elevated levels of DUSP6 expression such as malignant melanoma, papillary thyroid cancer, lung cancer, colorectal cancer, ovarian cancer, liver cancer, leukemia and a wide variety of other cancers.

Specifically, the present invention provides a method for inducing apoptosis in a melanoma tumor cell having a B-Raf mutation or exhibiting B-Raf hyperactivity and/or having a Ras kinase mutation or exhibiting Ras kinase hyperactivity by reducing DUSP6 activity. Preferably, the melanoma tumor cell is contacted with an agent that reduces DUSP6 activity. Such an agent is preferably a siRNA molecule, an antisense molecule, an antagonist, a ribozyme, an inhibitor, a peptide, or a small molecule. The agent may be a siRNA selected from Table 1 or Table 2. In the most preferred embodiment of the present invention, the agent is a siRNA molecule that comprises a polynucleotide selected from the group having a sequence of GUGCAACAGACUCGGAUGGUA, AGCUAACUGUGCGAUGAA, and the complements thereof.

The agent is preferably administered in a liposome, a nanoliposome, a ceramide-containing nanoliposome, a proteoliposome, a nanoparticulate, a calcium phosphor-silicate nanoparticulate, a calcium phosphate nanoparticulate, a silicon dioxide nanoparticulate, a nanocrystalline particulate, a semiconductor nanoparticulate, poly(D-arginine), a nanodendrimer, a virus, calcium phosphate nucleotide-mediated nucleotide delivery, by electroporation, or by microinjection.

The agent may be administered along with a chemotherapeutic agent selected from the group consisting of alkylating agents, antimetabolites, antibiotics, natural or plant derived products, hormones and steroids, and platinum drugs.

The present invention also provides a pharmaceutical composition for treating a melanoma tumor, having a B-Raf mutation or exhibiting B-Raf hyperactivity and/or having a Ras kinase mutation or exhibiting Ras kinase hyperactivity, comprising: an agent that reduces DUSP6 activity; and a carrier. Preferably, the carrier is selected from a group consisting of: a liposome, a nanoliposome, a ceramide-containing nanoliposome, a proteoliposome, a
nanoparticulate, a calcium phosphor-silicate nanoparticulate, a calcium phosphate nanoparticulate, a silicon dioxide nanoparticulate, a nanocrystalline particulate, a semiconductor nanoparticulate, poly(Darginine), a nanodendtimer, a virus, and calcium phosphate nucleotide-mediated nucleotide delivery. As stated above in relation to the method of the present invention, also in the pharmaceutical composition of the present invention the DUSP6 inhibiting agent is preferably a siRNA molecule, an antisense molecule, an antagonist, a ribozyme, an inhibitor, a small molecules or a peptide, that acts as a pseudosubstrate for DUSP6, such as a pseudosubstrate for a catalytic domain or a regulatory domain of DUSP6. Preferably the agent is an agent that acts as a competitive inhibitor for DUSP6, such as a competitive inhibitor for a catalytic domain of DUSP6 or an agent that results in downregulation of DUSP6 protein levels. The agent may be a siRNA selected from Table 1 or Table 2. In the most preferred embodiment of the present invention the agent is a siRNA molecule that comprises a polynucleotide selected from the group having a sequence of GUGCAACAGACUCGGAUGGUA, AGCUCAAUCUGUCGAUGAA, and the complements thereof.

Finally the present invention provides a method for detecting a cancer cell that is susceptible to this treatment, said method comprising: detecting a level of DUSP6 or fragment thereof in a test sample obtained from a cell of a subject, comparing the level of DUSP6 to a control level of DUSP6, wherein the presence of a cancerous cell is indicated by detection of an increased level of DUSP6 relative to the control.

In order to further increase the accuracy of the present method also the detection of BRAF and/or RAS mutations or increased levels of BRAF and/or RAS in conjunction with the increased DUSP6 level may be performed.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows exon structure of the two DUSP6 variants and location of the siRNAs.

Fig. 2 shows mRNA sequence of a long DUSP6 variant (NM_001946.2).

Fig. 3 shows mRNA sequence of a short DUSP6 variant (NM_022652.2).

Fig. 4 shows protein sequence of a long DUSP6 variant (NM_001946.2).

Fig. 5 shows protein sequence of a short DUSP6 variant (NM_022652.2).

Fig. 6 shows double stranded siRNA sequences.
Fig. 7 shows that DUSP6 mRNA levels are elevated in cancer cells (melanoma) with mutations resulting in hyperactivation of B-Raf and Ras kinases.

Fig. 8 confirms the knockdown of the DUSP6 protein due to treatment with siRNA-du1 and siRNA-du2.

Fig. 9 is a diagram displaying the relative expression-levels of DUSP6 mRNA in the same cancer cell line MaMel73b (melanoma) treated under identical conditions (25nM siRNA-du1, siRNA-du2 and siRNA Ctrl).

Fig. 10 shows differential effects on the viability of cancer cells (melanoma) with hyperactivation of Raf/Ras-MEK-ERK signalling pathways.

Fig. 11 shows selective effect of DUSP6 inactivation in cancer cells (melanoma) with hyperactivation of Raf/Ras-MEK-ERK signalling pathways and increased DUSP6 activity.

Fig. 12 shows that the reduction of cell viability is based on apoptosis in cancer cells (melanoma) with hyperactivation of Raf/Ras-MEK-ERK signalling pathways and increased DUSP6 activity.

Fig. 13 shows a time course of the apoptotic effect of DUSP6 inactivation in cancer cells (melanoma) with hyperactivation of Raf/Ras-MEK-ERK signalling pathways and increased DUSP6 activity.

Fig. 14 shows the effect of exemplarily selected siRNAs from table 1 on the viability of cells with B-Raf and DUSP6 hyperactivation (UKRV Ma-Mel 6b) and without B-Raf hyperactivation (MM Bank Ma-Mel 73b).

DETAILED DESCRIPTION OF THE INVENTION

Dual specificity phosphatase 6 (DUSP6, also known as MKP3 or PYST1) is well known as tumor suppressor (Zhang et al. 2010; Furukawa et al, 2003), thus at the present state of the art, the inactivation of DUSP6 is thought to promote tumorigenesis, which disqualifies a DUSP6 inactivation for use in cancer therapy. There are presently two DUSP6 transcripts known (Access, nos. NM_001946.2 and NM_022652.2; Fig 1-3), emerging by alternative splicing of exon 2 and giving rise to two protein variants (Fig. 4 and 5).

Two siRNAs targeting human DUSP6 were used as prototypes for inhibitors of DUSP6 activity (Fig. 1; Fig. 6). siRNA-du1 corresponded to duplex2, previously published to efficiently reduce DUSP6 protein levels (Zeliadt et al., 2008). siRNA-du2 corresponds to the
commercially available siRNA s4380 (Silencer® Pre-Designed & Validated siRNAs) from
Applied Biosystems, verified to reduce target mRNA levels by 70% or greater.

As an example to measure the increased DUSP6 activity in tumors, a set of 81 melanoma
(45 with hyperactivating B-Raf mutation, 18 with hyperactivating N-Ras mutation, 18 with
neither hyperactivating B-Raf nor hyperactivating N-Ras mutation) were analyzed for
elevated mRNA levels by quantitative RT-PCR (Fig. 7). Relative transcript levels of DUSP6
were assayed with the Hs00169257_m1 TaqMan® Gene Expression Assay (Applied
Biosystems) and normalized to the Endogenous Control Expression Assays for Human
GAPDH (Applied Biosystems). The following thermal cycling parameters were used: 50°C
for 2 min and 95°C for 15 min followed by 40 cycles of 95°C for 15 sec. and 60°C for 1 min
and results evaluated with the qbasePLUS evaluation software (Biogazelle). The levels of
the individual tumors were referred to the level of the matched normal tissue (NHEM;
Normal Human Epidermal Melanocytes, adult donor; PromoCell), which was set to 1. In this
example, the results from the individual tumors were averaged, which reveals a significant
upregulation of DUSP6 mRNA levels in both, tumors (melanoma) with hyperactivated B-Raf
kinase and/or hyperactivated Ras kinases (N-Ras).

As an example to achieve a reduction of DUSP6 activity the cells were cultured in RPMI
medium (Invitrogen) with 10% fetal calf serum, 2mM L-Glutamin, penicillin (100 U/mL) and
streptomycin (100 mg/mL) in a humidified atmosphere with 5% CO2 at 37°C. For the
knockdown- and viability studies, 12.500 cells per well were seeded into 24-well plates and
cultured over night. Afterwards, the medium was replaced with fresh medium and cells
transfected with 25nM siRNA using Lipofectamine RNAiMAX transfection reagent
(Invitrogen) according to manufacturer's protocol. The media was replaced 24h after
transfection and cells cultured for additional 96h before viability was assayed. Relative
numbers of viable cells were determined with the fluorometric CellTiter-Blue reagent
(Promega) and the fluorescence signal detected at the wavelengths 560/590 nm with a
Victor3 multilabel counter (PerkinElmer).

The reduction of the DUSP6 protein levels was confirmed by Western blotting in the cell line
MaMel73b (Fig. 8).

Fig. 8 is a confirmation of DUSP6 knockdown via siRNA-du1 and siRNA-du2. Protein
extracts were prepared from the cancer cell line MaMel73b (melanoma) after treatment with
25nM siRNA-du1 or siRNA-du2 and corresponding control treatment with siRNA-Ctrl. (Allstars control siRNA from Qiagen not targeting a cellular gene) and in addition also untreated cells without any treatment grown under normal conditions. Proteins were separated by SDS-PAGE and transferred by Western blotting. Afterwards, detection of DUSP6 was performed using the antibody MKP-3 (C-20) sc-8599 (goat polyclonal) from Santa Cruz Biotechnology. A beta-tubulin antibody (β-Tubulin (H-235) sc-9104; rabbit polyclonal from Santa Cruz Biotechnology) was used on the same membrane to confirm equal loading in the lanes. The following secondary antibodies were used: HRP-conjugated (Fab2)-fragment donkey anti-goat IgG (H+L) and Rabbit a-mouse IgG (H+L), both horseradish peroxidase conjugated from Jackson Immunoresearch and visualized using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology).

The same cancer cell line MaMel73b (melanoma) was confirmed for siRNA mediated silencing of DUSP6 mRNA via quantitative RT-PCR (Fig. 9).

The diagram in Fig. 9 displays the relative DUSP6 expression-levels of the same cancer cell line MaMel73b (melanoma) treated under identical conditions (25nM siRNA-du1 , siRNA-du2 and siRNA Ctrl, respectively as well as untreated sample). Total RNA was purified from the transfected cells and reversely transcribed. Relative transcript levels of DUSP6 were assayed with the Hs00169257_m1 TaqMan® Gene Expression Assay (Applied Biosystems) and normalized to the Endogenous Control Expression Assays for Human GAPD (GAPDH) and Human ACTB (beta actin) (Applied Biosystems). The following thermal cycling parameters were used: 50°C for 2 min and 95°C for 15 min followed by 40 cycles of 95°C for 15 sec. and 60°C for 1 min and results evaluated with the qbasePLUS evaluation software (Biogazelle).

Downregulation of DUSP6 by siRNA-du1 and siRNA-du2 is confirmed both on mRNA and Protein level.

Using a panel of cancer cell lines, melanoma cell lines, in this example with hyperactivating B-Raf mutation, differential effects on the viability of the cells can be observed (Fig. 10). Viability data are based on at least 3 independent assays.

The selective effect of DUSP6 inactivation is shown in Fig 11. The diagram displays the average relative DUSP6 expression levels of the different tumors (in this example
melanoma) in relation to the matched normal tissue (NHEM; Normal Human Epidermal Melanocytes, adult donor; PromoCell). GAPDH served as the reference gene for the determination of the mRNA levels. The top panel depicts the BRAF-mutation status (red/1: activating BRAF mutation, e.g. V600E mutation; green/0: no activating BRAF mutation), and the effects of siRNA-du1 and siRNA-du2 on the viability of the cancer cells. The numbers depict relative remaining cell viability compared to control-treated cells after treatment with the respective siRNA.

In another example to selectively kill cancer cells (melanoma) with hyperactivation of one of the Ras kinases, eg. N-Ras, the cells are cultured in RPMI medium (Invitrogen) with 10% fetal calf serum, 2mM L-Glutamin, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere with 5% CO₂ at 37°C. For the knockdown- and viability studies, 12,500 cells per well are seeded into 24-well plates and cultured over night. Afterwards, the medium is replaced with fresh medium and cells transfected with 25nM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to manufacturer’s protocol. The media are replaced 24h after transfection and cells cultured for additional 96h before viability are assayed. Relative numbers of viable cells are determined with the fluorometric CellTiter-Blue reagent (Promega) and the fluorescence signal is detected at the wavelengths 560/590 nm with a Victor3 multilabel counter (PerkinElmer).

The reduction of DUSP6 at the protein and mRNA level is determined as described above for the examples shown in Fig. 8 and 9.

The selective effect of DUSP6 inactivation in tumors with Ras (N-Ras in this example) kinase hyperactivation is determined in analogy to Fig 11. A reduction of cell viability equivalent to the one observed for tumors with B-Raf kinase hyperactivation and DUSP6 hyperactivation is observed for tumors with Ras (N-Ras) kinase hyperactivation and DUSP6 hyperactivation. Thus, a selective elimination of tumors with Ras kinase and DUSP6 hyperactivation can also be achieved.

The diagram in fig. 12 displays the caspase-3 and -7 activity of in relation to the viable cells 120 h after treatment with the DUSP6-inhibiting siRNA-du1 and siRNA-du2 in the responsive MaMel7 cells as an example for a tumor with B-Raf or Ras kinase hyperactivation (in this example B-Raf hyperactivation). From fig. 12, it can be seen that inhibition of DUSP6 eliminates cancer cells by inducing apoptosis.
The diagram in fig. 13 shows the caspase-3 and -7 activity of in relation to the viable cells 48h, 72h and 120 h after treatment with the DUSP6-inhibiting siRNA-du2 in the responsive UKRVMel6b and MaMel7 cells examples for tumors with B-Raf or Ras kinase hyperactivation (in this example B-Raf hyperactivation). From fig. 13, it can be seen that inhibition of DUSP6 eliminates cancer cells by inducing apoptosis.

For analyzing apoptosis, 3,000 cells per well were seeded into 96-well plates and cultured over night. Afterwards, the medium was replaced with fresh medium and cells transfected with 25nM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to manufacturer's protocol. The media was replaced 24h after transfection and cells cultured for additional 24h, 48h or 96h before investigation. Apoptosis was assayed due to the activity of Caspase-3 and -7 (key effectors in apoptosis in mammalian cells) using the ApoONE Homogeneous Caspase-3/7 assay (Promega) and fluorescence signal detected at the wavelengths 485/535 nm with a Victor3 multilabel counter (PerkinElmer). The Caspase signal was normalized to the relative cell number analyzed in parallel using the fluorometric CellTiter-Blue reagent (Promega) at conditions described above.

In another example to selectively kill cancer cells (melanoma) with hyperactivation BRAF, the cells are cultured in RPMI medium (Invitrogen) with 10% fetal calf serum, 2mM L-Glutamin, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere with 5% CO2 at 37°C. For the knockdown- and viability studies, 2,000 cells per well are seeded into 96-well plates and cells are transfected with 20 nM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to manufacturer's protocol and using one of the siRNAs listed in table 1. The media are replaced 24h after transfection and cells cultured for additional 96h before viability are assayed. Relative numbers of viable cells are determined with the fluorometric CellTiter-Blue reagent (Promega) and the fluorescence signal is detected at the wavelengths 560/590 nm with a Victor3 multilabel counter (PerkinElmer).

The reduction of DUSP6 at the protein and mRNA level is determined as described above for the examples shown in Fig. 8 and 9.

The selective effect of DUSP6 inactivation in tumors with B-Raf kinase hyperactivation is determined in analogy to Fig 11. A reduction of cell viability equivalent to the one observed
for siRNA-du1 and siRNA-du2 is observed for tumors with B-Raf kinase hyperactivation and DUSP6 hyperactivation. Fig. 13 shows exemplarily the effects of siRNA-du84, siRNA-du85, siRNA-du86, siRNA-du87 from table 1 and of a pool of these four siRNAs on the cell viability of the UKRV Ma-Mel 6b (with B-Raf kinase and DUSP6 hyperactivation) and MM Bank Ma-Mel 73b (without B-Raf hyperactivation) demonstrating that any siRNA randomly selected from table 1 would exert the effect.

Thus, a selective elimination of tumors with B-Raf kinase and DUSP6 hyperactivation can be achieved by any of the examples shown in table 1.

In another example to selectively kill cancer cells (melanoma) with hyperactivation of one of the Ras kinases, eg. N-Ras, the cells are cultured in RPMI medium (Invitrogen) with 10% fetal calf serum, 2mM L-Glutamin, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere with 5% CO2 at 37°C. For the knockdown- and viability studies, 2,000 cells per well are seeded into 96-well plates and transfected with 20nM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to manufacturer's protocol and one of the siRNAs depicted in table 1. The media are replaced 24h after transfection and cells cultured for additional 96h before viability are assayed. Relative numbers of viable cells are determined with the 25 fluorometric CellTiter-Blue reagent (Promega) and the fluorescence signal is detected at the wavelengths 560/590 nm with a Victor3 multilabel counter (PerkinElmer).

The reduction of DUSP6 at the protein and mRNA level is determined as described above for the examples shown in Fig. 8 and 9.

The selective effect of DUSP6 inactivation in tumors with Ras (N-Ras in this example) kinase hyperactivation is determined in analogy to Fig. 11 and Fig. 14. A reduction of cell viability equivalent to the one observed for tumors with B-Raf kinase hyperactivation and DUSP6 hyperactivation is observed for tumors with Ras (N-Ras) kinase hyperactivation and DUSP6 hyperactivation. Thus, a selective elimination of tumors with Ras kinase and DUSP6 hyperactivation can also be achieved with any of the siRNAs listed in table 1.

In relation to Table 1 it is important to note that the sequences in CAPITAL LETTERS constitute the core of the present invention. Thus, e.g. the indicated tt-overhangs may be
replaced with e.g. tc-overhangs or even eliminated. Table 2 shows siRNA precursors of the present invention.

| siRNA-du1 | sense: | 5'-GUGCAACAGACUCGGAUGGUAT\dagger.i-3' | SEQ ID NO 1 |
| anti-sense: | 3'-UACCAUCCGAGUCUGUUGCA\dagger-S` |
| siRNA-du2 | sense: | 5'-AGCUCAUCUGCGAUGAAT\dagger-3` | SEQ ID NO 2 |
| anti-sense: | 3'-UUCAUCAGACAUUGAC\daggertc-3` |
| siRNA-du3 | sense: | 5'-GAGACGCUCGCUGUUAUCCAU\daggertt-3` | SEQ ID NO 3 |
| anti-sense: | 3'-AAUGGAUACAAACAGCGAGC\daggerGUC-3` |
| siRNA-du4 | sense: | 5'-CAAGAGAUAGCAGGCGGUAACU\daggertt-3` | SEQ ID NO 4 |
| anti-sense: | 3'-UUAGACUCCGUAUUGCUAC\daggerUUt-3` |
| siRNA-du5 | sense: | 5'-GAGACGCUCGGCGAUAUCCAU\daggertt-3` | SEQ ID NO 5 |
| anti-sense: | 3'-AAUGGAUACGAACGAGC\daggerGUCU-3` |
| siRNA-du6 | sense: | 5'-CAAGAGAUAGCAGGCGGUAACU\daggertt-3` | SEQ ID NO 6 |
| anti-sense: | 3'-UUAGACUCCGUAUUGCUAC\daggerUUt-3` |
| siRNA-du7 | sense: | 5'-CAGCAGCGACGCGAGCAGCG\daggerGUGU-3` | SEQ ID NO 7 |
| anti-sense: | 3'-UAACCUUGUACAGACAGAT\daggerGUGU-3` |
| siRNA-du8 | sense: | 5'-GAGACGCUCCGCGCUGCU\daggerUUt-3` | SEQ ID NO 8 |
| anti-sense: | 3'-UUAGCUUUUAACUCGUC\daggerCUU-3` |
| siRNA-du9 | sense: | 5'-CAGCAGCGACGCGAGCAGCG\daggerGUGU-3` | SEQ ID NO 9 |
| anti-sense: | 3'-UAACCUUGUACAGACAGAT\daggerGUGU-3` |
| siRNA-du10 | sense: | 5'-CGCUGCUAGACAGACAG\daggerUUt-3` | SEQ ID NO 10 |
| anti-sense: | 3'-UAACCUUGUACAGACAGAT\daggerGUGU-3` |
| siRNA-du11 | sense: | 5'-UAGGUUUCGUGUACACCAUG\daggerUUt-3` | SEQ ID NO 11 |
| anti-sense: | 3'-UAACCAUCCGGUACAGCA\daggerCUU-3` |
| siRNA-du12 | sense: | 5'-CGCUCUGUACAGUG\daggerUUt-3` | SEQ ID NO 12 |
| anti-sense: | 3'-AACUGAUACCAAACUACG\daggerGUGU-3` |
| siRNA-du13 | sense: | 5'-CGCUCUGUACAGUG\daggerUUt-3` | SEQ ID NO 13 |
| anti-sense: | 3'-AACUGAUACCAAACUACG\daggerGUGU-3` |
| siRNA-du1 4 | sense: | 5'-CAGCAUCAGCUGGGCUGCUUUUtt-3' | SEQ ID NO 14 |
| anti-sense: | 5'-AAAGAAAGCAGCCACGCUGAUGCUtt-3' |
| siRNA-du1 5 | sense: | 5'-CAGCUGUCUGUACUAGACAAGGUUAtt-3' | SEQ ID NO 15 |
| anti-sense: | 5'-UAACCUUGCUAGUACAGACGCUUtt-3' |
| siRNA-du1 6 | sense: | 5'-UAGGUUUGCUCUGUACCCAGUGUAAtt-3' | SEQ ID NO 16 |
| anti-sense: | 5'-UCACAGCUUGCUAGCAACACCUUtt-3' |
| siRNA-du1 7 | sense: | 5'-AGGGCCUUGUAAGCGUGUUCCUUtt-3' | SEQ ID NO 17 |
| anti-sense: | 5'-AAAGGAACAGCCUUAACAGGCCTt-3' |
| siRNA-du1 8 | sense: | 5'-CAGCUCUGUAUUUGUGGGAUCGUtt-3' | SEQ ID NO 18 |
| anti-sense: | 5'-AACUGAUAACCCAAAUACAGGCUtt-3' |
| siRNA-du1 9 | sense: | 5'-GAGAUAGCAAAUCCGAGCUUUAtt-3' | SEQ ID NO 19 |
| anti-sense: | 5'-UAAGACUCGAUUUGCUACUtt-3' |
| siRNA-du20 | sense: | 5'-GGGAUUAGAAGCUCUAGACUUtt-3' | SEQ ID NO 20 |
| anti-sense: | 5'-AGUCUAGCGCUUACUACUtt-3' |
| siRNA-du21 | sense: | 5'-GGAUUAGAAGCCGCUAGACUtt-3' | SEQ ID NO 21 |
| anti-sense: | 5'-AAGUCUAGCGCUUACUACUtt-3' |
| siRNA-du22 | sense: | 5'-GGAUUAGAAGCCGCUAGACUtt-3' | SEQ ID NO 22 |
| anti-sense: | 5'-AAGUCUAGCGCUUACUACUtt-3' |
| siRNA-du23 | sense: | 5'-GGUAAGGGCGAGCGGAUUAAtt-3' | SEQ ID NO 23 |
| anti-sense: | 5'-UUAAUUGCGCCUCACUACUtt-3' |
| siRNA-du24 | sense: | 5'-GCAGCGACUGGAACGAAUAtt-3' | SEQ ID NO 24 |
| anti-sense: | 5'-UAUUCUGUCCAGACGCGUtt-3' |
| siRNA-du25 | sense: | 5'-GCAACAGACUGCGAUUGUAGUtt-3' | SEQ ID NO 25 |
| anti-sense: | 5'-ACUACCCGAGCUUGUGUtt-3' |
| siRNA-du26 | sense: | 5'-GGAGGAUUUCGGCAUGCAUGUAtt-3' | SEQ ID NO 26 |
| anti-sense: | 5'-UACUCUGCGAAUCCUACUtt-3' |
| siRNA-du27 | sense: | 5'-GAACCAAAUAGAUAGGGGAAGAttt-3' | SEQ ID NO 27 |
| anti-sense: | 5'-UCCUACCAAUUGUAACUACUtt-3' |
| siRNA-du28 | sense: | 5'-GUGAAGAAGGGAGAGUUAAAAtt-3' | SEQ ID NO 28 |
anti-sense: 5'-AUUUAACUCUUCCCUUCUCACtt-3'
siRNA-du29 sense: 5'-GAACCAAAUGAUAGGGUAGGAtt-3' SEQ ID NO 29

anti-sense: 5'-GUGAAGAGGGGAGAGUAAAUtt-3'
siRNA-du30 sense: 5'-AUUUAACUCUCCCUUCUCACAtt-3' SEQ ID NO 30

anti-sense: 5'-AGGCUGAGUCCAAGAGUAtt-3'
siRNA-du31 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 31

anti-sense: 5'-UAUCUCUUGGACUCAGCCUtt-3'
siRNA-du32 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 32

anti-sense: 5'-UGUCAAUGAAGACUACCUUtt-3'
siRNA-du33 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 33

anti-sense: 5'-UAUUUGCUAUCUCUUGGACUtt-3'
siRNA-du34 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 34

anti-sense: 5'-UCUCUCUCAAUGAAGCUGCtt-3'
siRNA-du35 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 35

anti-sense: 5'-UGAGAGAGAUUUUGAGGUGCAtt-3'
siRNA-du36 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 36

anti-sense: 5'-UGICAAGCAAAUCGAGUCUAtt-3'
siRNA-du37 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 37

anti-sense: 5'-AGUCUAGCGGCUUCUAAUtt-3'
siRNA-du38 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 38

anti-sense: 5'-AAUCCAAUUAAUUGGACUtt-3'
siRNA-du39 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 39

anti-sense: 5'-UCUCUCUCAAUGAAGCUGCtt-3'
siRNA-du40 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 40

anti-sense: 5'-UCUCUCUCAAUGAAGCUGCtt-3'
siRNA-du41 sense: 5'-AGUCCAAGAGAUAGCAAAUtt-3' SEQ ID NO 41

anti-sense: 5'-UGAGAGAGAUUUUGAGGAGAtt-3'
siRNA-du42 sense: 5'-UGUCAAUGAAUCUCUCACAttt-3' SEQ ID NO 42

anti-sense: 5'-AGAGAUUCAUGAGCACUAAAtt-3'
siRNA-du43 sense: 5'-AGAGAUUCAUGAGCACUAAAtt-3' SEQ ID NO 43
| siRNA-du44 | sense: 5'-GCAGCUGGGUGCAGAGAAtt-3' | 175x744 | seq id no 44 |
|           | anti-sense: 5'-UUAGUGUCAAUGAAUCUCtt-3' |
| siRNA-du45 | sense: 5'-AGAGAGAACCUCGGGCUUUt-3' | 175x744 | seq id no 45 |
|           | anti-sense: 5'-AAAGCCGGAGGUUCUCUtt-3' |
| siRNA-du46 | sense: 5'-GGGUAAAGCGCGGGAUAtt-3' | 175x744 | seq id no 46 |
|           | anti-sense: 5'-AUUCCGCCUCCGCUAAACCtt-3' |
| siRNA-du47 | sense: 5'-CGGAAAUGCCGAUCAGCAAtt-3' | 175x744 | seq id no 47 |
|           | anti-sense: 5'-UUGCUGAUCGCAUUUCCGtt-3' |
| siRNA-du48 | sense: 5'-GAAAGGGCGAUCAGCAAGAtt-3' | 175x744 | seq id no 48 |
|           | anti-sense: 5'-UCUUGCUGAUCGCAUUUCCtt-3' |
| siRNA-du49 | sense: 5'-CCGACACAGUGGUGCUCAttt-3' | 175x744 | seq id no 49 |
|           | anti-sense: 5'-UAGAGCACCACUGUGUCGtt-3' |
| siRNA-du50 | sense: 5'-UCAAGAAGCUCAAGGACGAAtt-3' | 175x744 | seq id no 50 |
|           | anti-sense: 5'-UCGUCUUGAGCUUCUGAAtt-3' |
| siRNA-du51 | sense: 5'-UGGCUUACCUUAUGCAGAAAtt-3' | 175x744 | seq id no 51 |
|           | anti-sense: 5'-UUCUGCAUAAGGUAAGCCAtt-3' |
| siRNA-du52 | sense: 5'-ACUUCAACUCUCAUGGGUGCAAtt-3' | 175x744 | seq id no 52 |
|           | anti-sense: 5'-UGACCCCAUGAAGUUGAAGUtt-3' |
| siRNA-du53 | sense: 5'-CUUCCAACAGAAUGUAAttt-3' | 175x744 | seq id no 53 |
|           | anti-sense: 5'-UAUACACAGUCCGUGGAAGAtt-3' |
| siRNA-du54 | sense: 5'-CUGCAACUCUCUGGAAGAAtt-3' | 175x744 | seq id no 54 |
|           | anti-sense: 5'-UCUUCAGCUGAGAUUGCAAtt-3' |
| siRNA-du55 | sense: 5'-CGGAAUUGCUUAAUACUAAtt-3' | 175x744 | seq id no 55 |
|           | anti-sense: 5'-UUAGUAAUACCAAUUCCGtt-3' |
| siRNA-du56 | sense: 5'-AGGACAUUGCUAUGUAAttt-3' | 175x744 | seq id no 56 |
|           | anti-sense: 5'-UAUCUAUCAGCAUGUCCUt-3' |
| siRNA-du57 | sense: 5'-AGAUAACAGGCUGAGGUUUt-3' | 175x744 | seq id no 57 |
|           | anti-sense: 5'-AAACCUAACUCUGCCGUAUUt-3' |
| siRNA-du58 | sense: 5'-CCAUGCGAGGGCAUGGAAUtt-3' | 175x744 | seq id no 58 |
siRNA-du59
sense: 5'-GGUAGGAGCAUGUGUUCUtt-3'
anti-sense: 5'-AAUCCCAGUCCCUGCAUGGtt-3'
SEQ ID NO 59

siRNA-du60
sense: 5'-GGAACUGACUAUAUAUUUGtt-3'
anti-sense: 5'-CAAUUUAUAAGUCAGUCCtt-3'
SEQ ID NO 60

siRNA-du61
sense: 5'-UGCAUAUGAGAGGCCAAtt-3'
anti-sense: 5'-UUUUGGCUCUCAUAUGGAtt-3'
SEQ ID NO 61

siRNA-du62
sense: 5'-UAGAGGAGGCAAGAGAGAtt-3'
anti-sense: 5'-UCUCUCUUUGGCUCUAtt-3'
SEQ ID NO 62

siRNA-du63
sense: 5'-UGACAGUGUUUGUUUGAAUtt-3'
anti-sense: 5'-AUUCAAACAAACACUGUAtt-3'
SEQ ID NO 63

siRNA-du64
sense: 5'-GAGGAGCCAAAGAGAGAUUtt-3'
anti-sense: 5'-UCUCCCUUUGGAUAAUUUtt-3'
SEQ ID NO 64

siRNA-du65
sense: 5'-GAAAUUAUCCAAAGGAGAtt-3'
anti-sense: 5'-UCUCCCUUUGGAUAAUUUtt-3'
SEQ ID NO 65

siRNA-du66
sense: 5'-AAAGGGAGAAGAGACAAAtt-3'
anti-sense: 5'-UCUCUCAAAACACUGUCAtt-3'
SEQ ID NO 66

siRNA-du67
sense: 5'-GAGAAAGAGACCAGUAUCCAt-3'
anti-sense: 5'-UCUCCCUUUGGAUAAUUUtt-3'
SEQ ID NO 67

siRNA-du68
sense: 5'-CAGUAUGCCACUUCAAAAtt-3'
anti-sense: 5'-UUUAAAGAGUGCCAGUACUGtt-3'
SEQ ID NO 68

siRNA-du69
sense: 5'-GGAUAAUCUGGAAAGGACAtt-3'
anti-sense: 5'-UGUCUUUCCCAAGAUUACCCtt-3'
SEQ ID NO 69

siRNA-du70
sense: 5'-GGGAAAGACACCCAAAUAUtt-3'
anti-sense: 5'-AUGAUUUUGUGGCUUCCCTtt-3'
SEQ ID NO 70

siRNA-du71
sense: 5'-AAUCAUGGGGCUCACUAUAAAtt-3'
anti-sense: 5'-UUAAAGUGAGCCCAUGAUUtt-3'
SEQ ID NO 71

siRNA-du72
sense: 5'-ACCCAUUUGUAAAGAGAAtt-3'
anti-sense: 5'-UUUCUCUUAUCAAUGGGAtt-3'
SEQ ID NO 72

siRNA-du73
sense: 5'-UGCAUUUGAUUGUGAAGAAtt-3'
SEQ ID NO 73
anti-sense: 5'-UUCUUCACAAUCAAAUGCAtt-3'
siRNA-du74
sense: 5'-UGAAGAAGGGAGAGUUAAAtt-3'
SEQ ID NO 74

anti-sense: 5'-CCAUUAUGUUGCUGGUGUAAtt-3'
siRNA-du75
sense: 5'-UACACCACGAACAUAAUGGtt-3'
SEQ ID NO 75

anti-sense: 5'-GCAUCAAGUACAUCAUUAAtt-3'
siRNA-du76
sense: 5'-UUCAAGAUGUACUGAGGtt-3'
SEQ ID NO 76

anti-sense: 5'-GAGUUUAAAUACAAGCAAAtt-3'
siRNA-du77
sense: 5'-GUUGCUUGUAUUUAAACUtt-3'
SEQ ID NO 77

anti-sense: 5'-GUGUGUAUUUAAACCAGtt-3'
siRNA-du78
sense: 5'-CUACUUGGACGUGUUGAtt-3'
SEQ ID NO 78

anti-sense: 5'-GUGGCUUGCUGUUGGACUtt-3'
siRNA-du79
sense: 5'-GCUACUUGGACGUGUUGCAtt-3'
SEQ ID NO 79

anti-sense: 5'-GUUCUUCACAAUCAAAUGCAtt-3'
siRNA-du80
sense: 5'-UGAAGAAGGGAGAGUUAAAtt-3'
SEQ ID NO 80

anti-sense: 5'-CUACUUGGACGUGUUGCAtt-3'
siRNA-du81
sense: 5'-UCUUGCCCUUCUCCUCUUCUtt-3'
SEQ ID NO 81

anti-sense: 5'-UCUUGCCCUUCUCCUCUUCUtt-3'
siRNA-du82
sense: 5'-UCUUGCCCUUCUCCUCUUCUtt-3'
SEQ ID NO 82

anti-sense: 5'-GCAGAGCCUCUUCUCCUCUUCUtt-3'
siRNA-du83
sense: 5'-UCUUGCCCUUCUCCUCUUCUtt-3'
SEQ ID NO 83

anti-sense: 5'-UUGGCUUACCUAUGCGAAtt-3'
siRNA-du84
sense: 5'-UCUUGCCCUUCUCCUCUUCUtt-3'
SEQ ID NO 84

anti-sense: 5'-UCUUGCCCUUCUCCUCUUCUtt-3'
siRNA-du85
sense: 5'-UCUUGCCCUUCUCCUCUUCUtt-3'
SEQ ID NO 85

anti-sense: 5'-GACUGUGGCUUACCUAUGGtt-3'
siRNA-du86
sense: 5'-GACUGUGGCUUACCUAUGGtt-3'
SEQ ID NO 86

anti-sense: 5'-GACUGUGGCUUACCUAUGGtt-3'
siRNA-du87
sense: 5'-GCCAGGACGAGAAUACtt-3'
SEQ ID NO 87
siRNA-du88  

sense: 5'-UCUUUGGUUAUCUAGCUAGCUAGUAUGA-3'  

anti-sense: 5'-AUAAAGCUAGAUAAACCGAAGU-3'

Table 2

| siRNA       | SEQ ID   | 5'-
|-------------|----------|------
| p-du89      | 89       | GGGGUUGGUUGUUAUCUUGGUUAUCUAGCUAGCUAGGUUGUGUGAGUCUCAUAAA
|             |          | GCUAGAUAAACCGAAGUAAACUGAACCUC-3' |
| p-du90      | 90       | GGAAGCGAGUUGUUAUCUUGGUUAUCUAGCUAGCUAGGUUGUGAGUGCUCAUAAAGC
|             |          | UAGAUAAACCGAAGUAAACUGAACCUC-3' |
| p-du91      | 91       | GGAGGCCCUGUCUCUUGGUUAUCUAGCUAGCUAGUGCGACAGACGCGCUCAUAAA
|             |          | GCUAGAUAAACCGAAGUAAACUGAACCUC-3' |
REFERENCES


CLAIMS

1. An agent that reduces DUSP6 activity for use in inducing apoptosis in cancer cells having a B-Raf mutation or exhibiting B-Raf hyperactivity.

2. The agent for use as in claim 1, wherein the cells have increased DUSP-6 activity.

3. The agent for use as in claim 1 or 2, wherein the cells are melanoma cells.

4. The agent for use as in claim 3, wherein the agent is selected from the group consisting of a siRNA molecule, an antisense molecule, an antagonist, a ribozyme, an inhibitor, a peptide, a polypeptide, or a small molecule.

5. The agent for use as in claim 4, wherein the agent is a siRNA selected from Table 1 or Table 2.

6. An agent that reduces DUSP6 activity for use in inducing apoptosis in cancer cells having a Ras kinase mutation or exhibiting Ras kinase hyperactivity.

7. The agent for use as in claim 6, wherein the cells have increased DUSP-6 activity.

8. The agent for use as in claim 6 or 7, wherein the cells are melanoma cells.

9. The agent for use as in claim 8, wherein the agent is selected from the group consisting of a siRNA molecule, an antisense molecule, an antagonist, a ribozyme, an inhibitor, a peptide, a polypeptide, or a small molecule.

10. The agent for use as in claim 9, wherein the agent is a siRNA selected from Table 1 or Table 2.

11. A pharmaceutical composition for treating a tumor, having a B-Raf and/or Ras kinase mutation or exhibiting B-Raf and/or Ras kinase hyperactivity, and preferably having increased DUSP6 activity, comprising: an agent that reduces DUSP6 activity and a carrier.
12. The pharmaceutical composition of claim 11, wherein said carrier is selected from a group consisting of: a liposome, a nanoliposome, a ceramide-containing nanoliposome, a proteoliposome, a nanoparticulate, a calcium phosphor-silicate nanoparticulate, a calcium phosphate nanoparticulate, a silicon dioxide nanoparticulate, a nanocrystalline particulate, a semiconductor nanoparticulate, poly(Darginine), a nanodendrimer, a virus, and calcium phosphate nucleotide-mediated nucleotide delivery.

13. The pharmaceutical composition of claim 11 or 12, wherein said agent is selected from the group consisting of: siRNA molecule, an antisense molecule, an antagonist, a ribozyme, an inhibitor, a peptide, a polypeptide, or a small molecule.

14. The pharmaceutical composition of claim 11, wherein the agent is a siRNA selected from Table 1 or Table 2.

15. The pharmaceutical composition of claim 11 wherein said small interfering RNA (siRNA) molecule comprises: 5'-GUGCAACAGACUCGGAUGGUAtt-S or 5'-AGCUCAACUUGCUAGAAtt-3' or the complement thereof.

16. A small interfering RNA (siRNA) molecule selected from Table 1 or Table 2 for use as a cancer medicament.

17. A small interfering RNA (siRNA) molecule selected from Table 1 or Table 2, such as 5'-GUGCAACAGACUCGGAUGGUAtt-S or 5'-AGCUCAACUUGCUAGAAtt-3' for use in the treatment of malignant melanoma.

18. A method for detecting a cancer cell responsive to the pharmaceutical composition of claim 11, said method comprising: detecting a level of DUSP6 or fragment thereof in a test sample obtained from a cell of a subject, comparing the level of DUSP6 to a control level of DUSP6, wherein the presence of a cancer cell responsive to the pharmaceutical composition of claim 13 is indicated by detection of an increased level of DUSP6 relative to a control level of DUSP6.

19. A method according to claim 18, wherein the method further involves detecting a B-Raf hyperactivating mutation or an increased level of B-Raf in the test sample.
20. A method according to claim 18 or 19, wherein the method further involves detecting a Raf kinase hyperactivating mutation or an increased level of Ras kinase in the test sample.
Fig. 1
1 gagagcctcg ctggattgtat ccattgagga gctgcctgc gcgaggggtg tggcagaggc
gagacagag atagccaa atcaatctaa gatcgggg ggagggcg agaagccgg ccgggttagat
121 tgtagggtaga gctctaggag gaggattag aacgcgttag acctttttttt ctccccctctc
gtagagcaat ccgagtattt catcaggtt cttagggacta caaatatacat gtaggcgtat
214 gacgccctca cgtagcgttcc gatggttact ttttcgctg cattccctcg ctgatcagc
ggcccccc ggtttgta caattttttt ctttttttttt ccttttttttt ctttttttttt
303 ggcacgtcag cgtggtccct acggcgttct cttggtttgtgc cctgtttttt gcacgttaa
391 ggtttttttt ttcttttttt ttcttttttt ttcttttttt ttcttttttt ttcttttttt
421 cttttttttt tttttttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
481 acaggtgcgg cggcgcgg cggcgcgg cggcgcgg cggcgcgg cggcgcgg
541 ggcgagcgct ggcgagcgct ggcgagcgct ggcgagcgct ggcgagcgct ggcgagcgct
601 gcggcggcgg cggcgcgg cggcgcgg cggcgcgg cggcgcgg cggcgcgg
661 atcagctg gcggcgcgggc ggcgagcgct ggcgagcgct ggcgagcgct ggcgagcgct
721 gagacagag acggcagag acggcagag acggcagag acggcagag acggcagag
781 acggcagag ggacggcagag ggtttgggtg ccttttttttt ttttcttttttt ttttcttttttt
841 aagagacggc gggggttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
901 gccgttgttct gctgtttttt ttcttttttt ttcttttttt ttcttttttt ttcttttttt
961 cggggttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
1021 gtagttgattt ctttttttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
1081 aacagggcgtt gctcttttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
1141 gacggccacac gttggagcc agttgactt ttttttttttt ttttttttttt ttttttttttt
1201 aataaattttt gtttttttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
1261 ggtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Fig. 3
MIDTLRPVPFASEMALKTVAVLNEQLGNERLLLMDCRPQELYESSHIESAINVAIP
GIMLRLQKGNLPVRALFTRGEDRDRFRRRCGTDTVLYDESSSDWNENTGGESVL
GLLLLKLIDGCRAFYLEDEARGKNCGVLVHCAGISRSVTVTAYLMQKLNSMND
AYDIVKMKKSISPNFNMQGLLDFERTLGLSSPCDNSRVPAPQLYFTTPSNQNYQV
DSLQST

Fig. 5
| siRNA-du1    | sense: | 5’-GUGCAACAGACUCGGAUGGUAtt-3’ |
|             | anti-sense: | 5’-UACCAUCCGAGUCUGUUGCACtt-3’ |
| siRNA-du2   | sense: | 5’-AGCUAAUCUGUCGAUGAAAtt-3’ |
|             | anti-sense: | 5’-UUCAUCGACAGAUUGAGCUtc-3’ |

**Fig. 6**
Fig. 7
Fig. 9

The chart shows the relative expression level of DUSP6 for different conditions:

- siRNA Ctrl: 100.00
- Untreated: 116.67
- siRNA-du1: 19.62
- siRNA-du2: 25.22
Fig. 10
Fig. 11
### A. CLASSIFICATION OF SUBJECT MATTER

**IPC:** A61K 31/7088 (2006.01), A61P 35/00 (2006.01), C12Q 1/68 (2006.01)

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

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

**IPC:** A61K, A61P, C12Q

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>P, X</td>
<td>MESSINA et al., &quot;Dual-specificity phosphatase DUSP6 has tumor-promoting properties in human glioblastomas&quot;, Oncogene, 2011, Vol. 30, No. 35, pages 3813-3820 See abstract</td>
<td>1-4, 6-9, 11-16</td>
</tr>
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* Further documents are listed in the continuation of Box C. See patent family annex.

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**Date of the actual completion of the international search**

23/01/2012

**Date of mailing of the international search report**

12/03/2012

**Name and mailing address of the ISA/Nordic Patent Institute**

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<table>
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<tr>
<td>A</td>
<td>OUYANG et al., &quot;Inhibitors of Raf kinase Activity Block Growth of Thyroid Cancer Cells with RET/PTC or BRAF Mutations In vitro and In vivo&quot;, Cancer Therapy: Preclinical, 2006, Vol. 12, No. 6, pages 1785-1793 See abstract</td>
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<tr>
<td>A</td>
<td>WO2009062199 A1 (FOX CHASE CANCER CENTER) 2009.05.14 See claim 18</td>
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</table>
### Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [ ] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   
   - [ ]

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   
   - [ ]

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

**See extra sheet**

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

   - [ ]

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   - 1-17

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

- [ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.
Continuation of Box III

Invention A (claims 1-17): an agent that reduces DUSP6 activity for use in inducing apoptosis in cancer cells; a pharmaceutical composition comprising an agent that reduces DUSP6 activity for treating a tumor; and a small interfering RNA (siRNA) molecule for use as a cancer medicament.

Invention B (claims 18-20): a method for detecting a cancer cell responsive to the pharmaceutical composition of invention A. The method comprises: detecting a level of DUSP6 or fragment thereof in a test sample obtained from a cell of a subject, comparing the level of DUSP6 to a control level of DUSP6, wherein the presence of a cancer cell responsive to the pharmaceutical composition of invention A is indicated by detection of an increased level of DUSP6 relative to a control level of DUSP6.

Use of an agent that reduces DUSP6 activity in treatment of cancer and a method for detecting a cancer cell responsive to said treatment are not found so linked as to form a single general inventive concept and there is a lack of unity between the two inventions.

Therefore, the requirement of unity is not fulfilled, according to Rule 13.2 PCT.

The first invention identified is invention A (claims 1-17).
<table>
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<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
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<td>WO2009062199 A1</td>
<td>20090514</td>
<td>US2010239656 A1</td>
<td>20100923</td>
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Form PCT/ISA/210 (patent family annex) (July 2009)