SHORT REPORT

Selective RNAi-mediated inhibition of mutated *c-kit*

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ABSTRACT

The proto-oncogene *c-kit* plays an important role in the development and survival of mast cells. Gain-of-function mutations in c-*kit* are one of the most characteristic events in mast cell leukemia (MCL) but as yet there is no clinically approved treatment for the disease. Here we describe growth inhibition of human MCL cell lines by the use of RNAi against *c-kit* or its mutant form. Retroviral transduction of HMC1.1 and HMC1.2 cell lines with vectors carrying DNA to be transcribed to RNAi against the wild type or mutant c-*kit* messengers reduced Kit protein levels considerably, decreased cell proliferation, and increased the apoptotic levels five days after retroviral infection. Thus RNAi targeted against Kit or its mutant form could be considered as a new antiproliferative agent against human mast leukemia cell lines, especially HMC1.2 cells which are resistant to the Kit tyrosine kinase inhibitor, imatinib mesylate.

KEYWORDS: RNAi, c-*kit*, allelic discrimination, mast cell leukemia (MCL), apoptosis

INTRODUCTION

The *c-kit* proto-oncogene codifies for a tyrosine kinase receptor that is essential for the proliferation and survival of hematopoyetic stem cells, melanocytes, insterstitial cells of Cajal, germinal cells and mastocytes. Mutations in *c-kit* have been associated with several diseases, such as gastrointestinal stromal tumours (GISTs) and mast cell leukemia (MCL) (Orfao et al, 2007), a rare form of mastocytosis characterized by the infiltration of large burdens of tissue mast cells into bone marrow and peripherical blood. Allele-specific gain-of-function mutations either at the juxtamembrane or the catalytic domain of c-*kit* have been found in MCL (Longley et al, 2001). Imatinib mesylate, the clinically approved c-Kit tyrosine kinase inhibitor, is able to block the survival of cells from the human leukemia mast cell line HMC1.1 as well as some cases of both MCL and GISTs, all of them harbouring mutations in the juxtamembrane domain of the protein (Hartmann et al, 2005). The drug nevertheless has no effect on the survival of MCL cells harbouring mutations at the catalytic domain of c-Kit, such as the human leukemia MCL line HMC1.2 (Akin et al, 2003). Additionally, tumours harbouring c-*kit* mutations that respond to imatinib mesylate, may develop new point mutations that confer them resistance to the drug (Haller et al, 2007; Nakagomi and Hirota, 2007).

RNAi has the potential for treatment of disorders where dominant gene mutations contribute to the progression of the disease (Kim and Rossi, 2007). In this report, we have investigated the effect of using shRNA to silence Kit in both the imatinib-sensitive HMC1.1 and the imatinib-resistant HMC1.2 cell lines. We found that RNAi may be used as an alternative treatment for MCL.

MATERIALS AND METHODS

Cell culture

HMC 1.1 and HMC1.2 cell lines were a generous gift from Drs Luis Escribano (Hospital Virgen del Valle, Toledo, Spain) and David Butterfield (Mayo Clinic, Rochester,

USA), respectively. Cells were grown in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) foetal bovine serum and 100µg/ml penicillin. The Phoenix amphotropic cell line was cultured in DMEM supplemented with 10% (v/v) foetal bovine serum.

Antibodies and reagents

Rabbit polyclonal anti-Kit (sc-168) was obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-actin secondary antibody was obtained from Sigma-Aldrich, Inc. STI-571 (Imatinib mesylate) was a gift from Novartis; working stocks were prepared by dissolving it in PBS at 1 mM and stored at -20ºC.

Retroviral-based Kit knockdown by shRNAs

We followed several previously described methods (Brummelkamp et al, 2002) to generate interfering RNAs against wild type and mutant (Val560Gly, Asp816Val) c-*kit* messengers, present in HMC1.1 and HMC1.2 cells. Short-hairpin DNA sequences selected from GenBank accession number NM_00222 were synthesised and cloned between the *Bgl*II and *Xho*I restriction endonuclease sites of the pSUPERretro vector that carries the puromycin resistance *pac* gene. Five shRNA-encoding retroviral vectors were tested in HMC1.1 and HMC1.2 cells.

Retroviral infection of human leukemia mast cells

Plasmid-retroviral vectors carrying *pac gene* (as selectable marker) and one of the cloned DNAs encoding Tot shRNAs, Mut shRNAs, non-allele specific shRNAs were transfected into Phoenix amphotrophic packaging cell lines using lipofectamine and Plus Reagent according to the manufecturer's instructions. Empty vector was used as non-shRNA expressing control. Forty-eight hours after transfection, viral supernatants were colleted and used to infect HMC1.1 and HMC1.2 cell lines in the presence of polybrene (8 µg/ml). Cells were incubated overnight and then allowed to recover for 24 hr in fresh medium. Twenty-four hours after retroviral infection, puromycin was added to cell cultures.

Quantitative RT-PCR

Five days after infection of HMC1.1 cells by empty retroviral vector (control) or by one of the shRNA-expressing vectors (Mut 1, Mut 2, Mut 3) total RNA was isolated using the RNAeasy Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), followed by single PCR reactions using the appropriate primer pairs for the amplification of either *c-kit* wild type or mutant alleles. Quantitative PCR assays were performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using Sybergreen. As PCR controls, we used RNA isolated from NCI-H510 human lung cancer cells (which carry wild-type *c-kit*) (Carney et al, 1985) and human RNA from HMC1.2 cells (which carry c-*kit* Val560Gly/Asp816Val mutant). The amplified products were isolated and cloned into pGEM-T vector. The PCR cycling conditions were: 35 cycles at 95ºC for 30 sec, 60ºC for 30 sec and 72ºC for 30 sec. The following primers were used in PCR amplification:

Forward primer for the wild type allele:
5' CATGTATGAAGTACAGTGGAAGGTT CT

Forward primer for the Val560Gly mutant allele:
5'-ATGTATGAAGTACAGT GAAGGTTCG

Reverse primer for both wild type and mutant alleles:
5'-GGAAGTTGTGTTG GGTCTATGTAAAC

Western blot analysis

Cells were harvested in TNSEV lysis buffer (50mM Tris-HCl pH7.5; 2 mM EDTA; 2mM sodium orthovanadate, 1%, v/v, NP-40) supplemented with 1 mM PMSF, 1 µg/ml leupeptin and 1 µg/ml aprotinin, and incubated on ice for 15 min. The homogenate was centrifuged for 15 min at 14000 rpm and the supernatant was taken for protein quantification. Protein samples (70 µg) were denatured in Laemmli buffer at 95ºC, and separated in 8% (v/v) and 10% (v/v) SDS-PAGE gel at 100 V for 2 hr, and electrophoretically transferred onto nylon membranes. The membranes were incubated for 1 hr with the appropriate primary antibodies dissolved in 5% (w/v) powdered non-fatty milk and 1% (v/v) Tween-20 in PBS. After washing, membranes were incubated for 45 min with the correspondent secondary antibodies. The bound antibodies were detected by chemiluminiscence using ECL reagents.

Flow cytometric apoptosis assay

The percentage of cell death was determined according to the supplier's instructions (BD, Pharmingen). After retroviral infection, HMC1.1 and HMC1.2 cells were washed in PBS and resuspended in 100 µl of annexin/7-AAD binding buffer and incubated for 15 min at room temperature in dark. Thereafter, 400µl of binding buffer was added to each sample and analyzed by flow cytometry using a FACS Calibur cytometer (BD, Heinderg, Germany).

Cell viability assays

These were based on trypan blue exclusion or 3,4-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide (MTT) assay. For the former, infected cells were washed in PBS, and resuspended in 1 vol of PBS and an equal volume of trypan blue. After 1 min incubation viable cells, represented as white spots, were counted using a Neubauer chamber under a light microscope. For the MTT assay, 3,4-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide (MTT) was dissolved in PBS at 1 µg/µl. Viable cells converted the MTT to a blue formazan precipitate. After 2 hr incubation at 37ºC in the dark, dark-blue formazan was solubilised with 2ml of DMSO and the absorbance at 540nm was measured.

Statistical analysis

Values are expressed as mean ± SD; comparisons were made using Student's t-test.

RESULTS

Knockdown of wild type and mutant Kit proteins by allele-specific shRNAs

We designed five different shRNAs targeting different regions of *c-kit* messengers. As shown in Figure 1A,

HMC1.1 cells had an allele-specific point mutation at position-1680 of c-*kit* cDNA, which corresponds to a Val560Gly substitution, and HMC1.2 cells had two different allele-specific point mutations, one at position 1680 and the other one at position 2448 of the same allele of c-*kit*, which corresponds to a Val560Gly and Asp816val substitution as described previously in the literature (Akin C et al, 2003). Two shRNAs targeted c-*kit* mRNA outside the mutated region inhibiting the expression of both mutant and wild type mRNAs (Total 1 and Total 2). Other two shRNAs were directed against the V560G mutant messenger of *c-kit* present in both HMC1.1 and HMC1.2 cells (RNAi Mut 1, RNAi Mut 2), and finally there was one non-specific allelic RNAi (RNAi Mut 3). Both HMC1.1 and HMC1.2 cell lines were infected with either the empty control retrovirus or the retroviral-based shRNAs. Five days after retroviral infection and puromycin selection of HMC1.1 and HMC1.2 cells, we tested the efficiency of retroviral-based shRNAs on Kit protein levels (Figure 1B). Total 2 RNAi against both wild type and mutant messengers of the gene, gave rise to 86% knockdown in HMC1.1 and 95% protein knockdown in HMC1.2, respectively. The Mut 1 RNAi against the mutant messenger V560G inhibited 50 and 58% of the total protein levels in HMC1.1 and HMC1.2, respectively and the Mut 2 RNAi did so for 52% and 57% of Kit protein. The non-allele specific RNAi (Mut 3 RNAi) inhibited Kit protein levels not only in HMC1.2 cells but also in HMC1.1 cells. The empty retroviral vector control did not show any significant reduction of Kit protein.

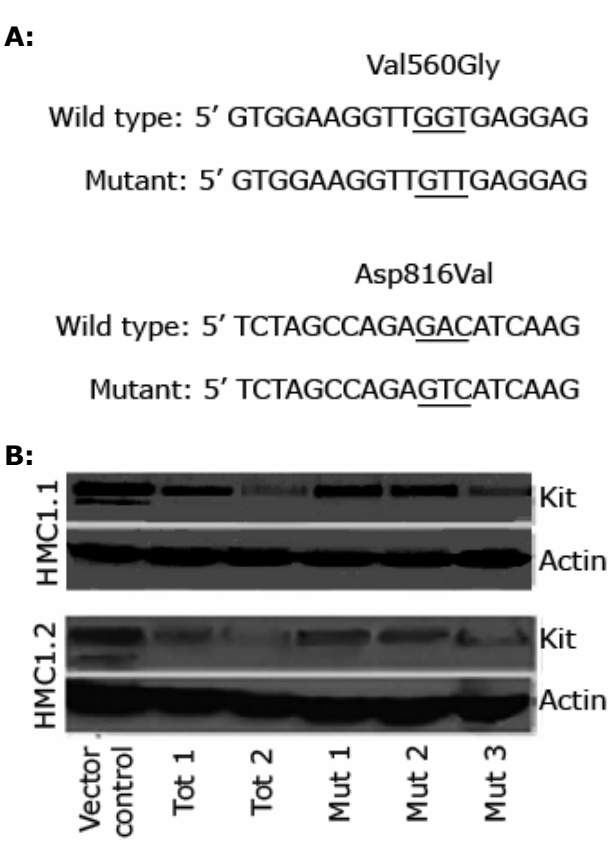

Figure 1. Localization of *c-kit* point mutations. **(A)** Identification of allele-specific c-*kit* point mutations in HMC1.1 and HMC1.2 cell lines after amplification by RT-PCR and sequencing. The G/T polymorphism for Val560Gly mutation and the A/T polymorphism for Asp816Val mutation in *c-kit* are underlined. **(B)** Western blot analysis showing the amount of Kit protein after transduction of HMC1.1(upper panel) and HMC1.2 (lower panel) cells with retroviruses carrying an empty retrovirus (Control vector) and with retroviruses expressing shRNA against: both messenger RNAs of c-*kit* (Tot1, Tot2); the mutated messenger RNAs (Mut1, Mut 2), or a non-allele specific shRNA (Mut 3). Actin was used as protein loading control.

Quantification of c-*kit* mutant transcripts after RNAi expression in HMC1.1 cells

Next, we performed a quantitative RT-PCR to confirm the efficacy and specificity of the RNAi directed against the mRNA of the mutant allele of *c-kit*. We designed specific primers that amplified only the mutant or the wild type *c-kit* cDNA (Figure 2A). The confirmation of the strict allelic discrimination of these primers was examined in a test experiment primed with either wild type or mutant cDNAs. As shown in Figure 2B wild type but not mutant *c-kit* cDNA was amplified in the presence of the wild type primers, while conversely mutant but not wild type cDNA was amplified in the presence of the mutant primers. Figure 2B also shows the amplification of both wild type and mutant *c-kit* cDNAs in retrotranscribed mRNA prepared from HMC1.1 and HMC1.2 cells, with both wild type and mutant primers.

Some but not all of the shRNAs differentially suppressed the wild type and mutant *c-kit* mRNAs. The results obtained were normalised with respect to those obtained with cells infected with an empty retrovirus. The levels of *c-kit* mutant mRNA in HMC1.1 cells treated with the Mut 1 and Mut 2 RNAi were reduced relative to wild type mRNA by 93% and 79%, respectively, indicating the specificity of these two shRNAs for the mutant messenger of *c-kit* (Figure 2C). Nevertheless, when cells were treated with Mut 3 RNAi, the ratio of mutant to wild type *c-kit* mRNAs was similar to that observed in cells infected with the control empty retrovirus, in agreement with our previous results in which Mut 3 RNAi inhibited mutant and wild type mRNAs in HMC1.1 cells indiscriminately.

Effects of Kit knockdown in HMC1.1 and HMC1.2 cells

Knowing that Kit is important for the proliferation and survival of mast cells, we tested whether RNAi mediated *c-kit* knockdown reduced mast cell survival. We used in most of the experiments retrovirus transcribing Mut 1 RNAi, which was the most efficient in the reduction of the mutant mRNAs of *c-kit,* and in some others Tot 2 RNAi, which reduced both forms of Kit indiscriminately. Twelve days after retroviral infection and puromycin selection, the proliferation of mast cells was markedly reduced by both retroviruses. Both total Kit knockdown (Tot) and mutant Kit knockdown (Mut) effectively reduced HMC1.1 and HMC1.2 cell viability (Figure 3A and B, respectively). To evaluate whether the decrease in cell viability was associated to apoptosis, we measured the percentage of annexin-V positive cells 5 days after infection. The death following infection in the two cell lines represented 27.97% for HMC1.1 and 16.05% for HMC1.2, respectively as compared to cells infected with the control

empty retrovirus (Figure 3C). We also found that the inhibition of only mutant *c-kit* mRNA showed even higher levels of apoptosis, being 44.64% and 27.63% in HMC1.1 and HMC1.2 cells, respectively.

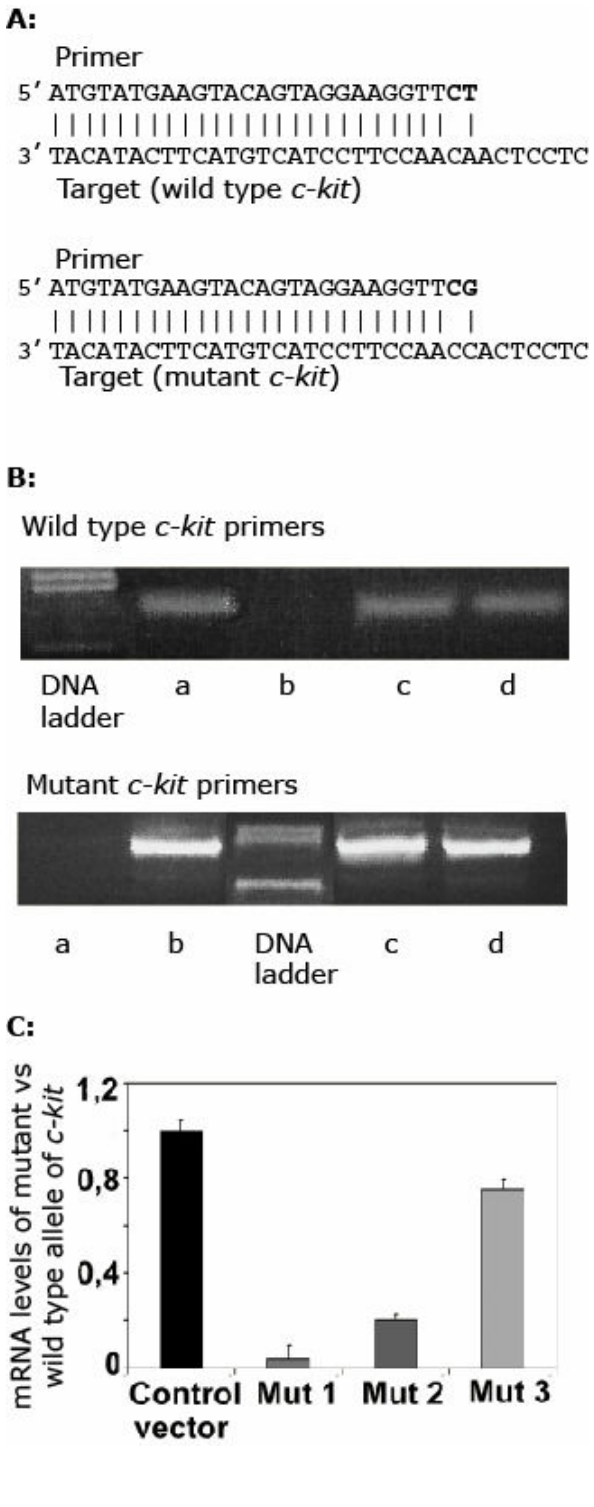

Figure 2. Selective knockdown of *c-kit* mutant messengers in HMC1.1 cells. **(A)** Sequence details of *c-kit* wild type and mutant cDNAs. **(B)** Agarose gel detection of amplified wild type or mutated *c-kit* cDNAs by standard PCR, using the wild type primers for the upper part of the figure and the mutant primers for the lower part: lane a, DNA amplified from cloned wild type *c-kit* originally from NCI-H510 lung cancer cells; lane b, DNA amplified from cloned mutant *c-kit* originally from HMC1.2 cells; lane c, product of RT-PCR amplification of RNA from HMC1.1 cells; d: product of RT-PCR amplification of RNA from HMC1.2 cells. **(C)** Estimated ratio of mutant to wild type mRNAs by qRT-PCR using sybergreen in HMC1.1 cells after treatment with control empty retrovirus, and retroviruses transcribing Mut 1 RNAi, Mut 2 RNAi and Mut 3 RNAi.

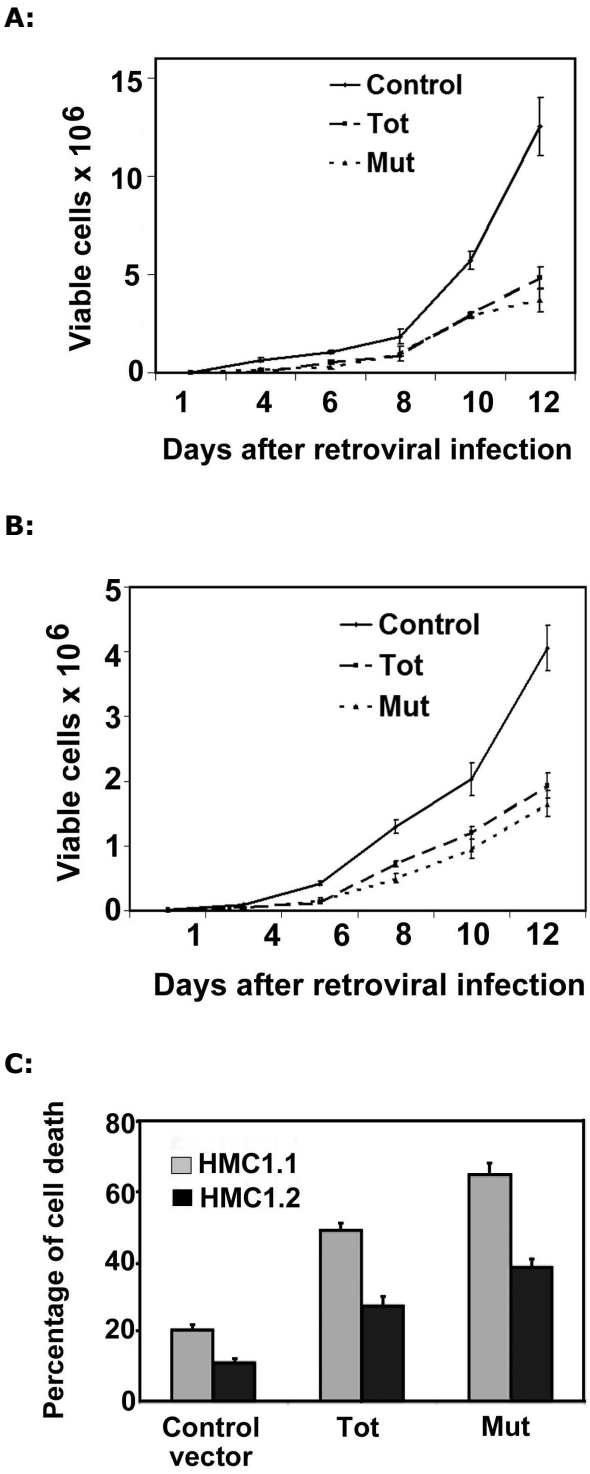

Figure 3. Cell survival of human mast leukemia cells after Kit knockdown. **(A)** Survival assay of HMC1.1 cells after gene silencing of all *c-kit* mRNAs (Tot) or only the mutant mRNA (Mut) at different times after retroviral infection. **(B)** Survival assay of HMC1.2 cells as in A. **(C)** Percentage of HMC1.1 (grey) and HMC1.2 (black) apoptotic cell death 5 days after Kit knockdown (annexin-V positive cells).

Imatinib mesylate resistant HMC1.1 cell line is sensitive to shRNA-mediated c-*kit* gene silencing

Imatinib mesylate is a potent tyrosine kinase inhibitor of Kit, PDGFR, and the fusion protein ABL-BCR (Kovalenko et al, 1994; Druker et al, 1996; Heinrich et al, 2000). We treated both HMC1.1 and HMC1.2 MCL cell lines with imatinib mesylate and compared the results with the ones obtained by shRNA-mediated *c-kit* gene silencing (Figure 4), showing as expected, that the drug drastically reduced cell viability in HMC1.1, but not in HMC1.2 cells as previously described, (Akin et al, 2003). On the other hand, Tot and Mut sh-RNAs silencing c-*kit* reduced considerably the viability of both HMC1.1 and HMC1.2 cells.

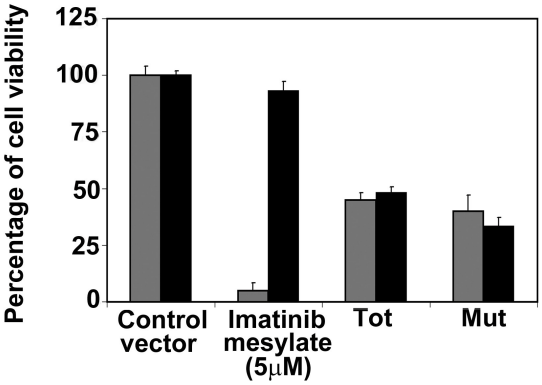

Figure 4. HMC1.2 cell line is resistant to imatinib mesylate but sensitive to Kit knockdown. The viability of HMC1.1 and HMC1.2 cells is shown 5 days after the various treatments shown at the bottom of the graph that are: empty vector (Control vector), imatinib mesylate, RNAi against total *c-kit* mRNAs (Tot) and RNAi against the mutant allele of *c-kit* mRNA (Mut).

DISCUSSION

Kit is up-regulated in several malignant diseases such as myeloproliferative disorders (Beghini et al, 2002), gastrointestinal stromal tumours (Hornick et al, 2007) and neuroblastomas (Vitali et al, 2003) although its role in the progression of the different types of cancer is not clearly established (Sattler and Salgia, 2004).

We have knockdown Kit protein in both HMC1.1 and HMC1.2 cell lines with RNAi. The ability of the RNAi to discriminate between the mutant and wild type messengers of *c-kit* has also been shown. Similar discriminatory inhibition has been demonstrated for other genes such as *p53*, *k-ras* and SOD-1, whose mutations are related to the progression of Li-Fraumeni syndrome, pancreatic cancer and amyotrophic lateral sclerosis respectively (Martinez et al, 2002; Duursma and Agami, 2003; Raoul et al, 2005). The expression of RNAi specific for the mutant form of *c-kit* resulted in a reduction of the corresponding mRNA in HMC1.1 cells of up to 93%, and also to a greater decrease in cell viability than when both mutant and wild type forms of the mRNA were inhibited. These results confirm the possibility of using allele discrimination to alleviate illness caused by negative dominant mutations.

Both cell lines HMC1.1 and HMC1.2 were responsive to the anti-proliferative effects of either total *c-kit* RNAi or mutant *c-kit* RNAi in a time-dependent manner. The observed apoptotic cell death is probably a result of the down-regulation of Bcl-2 and consequent reduction in the activation of downstream effectors such as Akt, Stat3 and Erk1/2, which are necessary for the normal development of mast cells as previously described (Ning et al, 2001; Nishida et al, 2002; Wandzioch et al, 2004). Mast leukemia HMC1.1 and HMC1.2 cell lines are good models to study the susceptibility of mutant *c-kit* variants to tyrosine kinase inhibitors such as imatinib mesylate. The HMC1.2 cell line, as shown here and previously (Akin et al, 2003), is resistant to imatinib mesylate as a consequence of a point mutation at D816V, the catalytic domain of the protein, while HMC1.1 harbouring exclusively the V560G point mutation is sensitive to the drug. The highly specific effect of RNAi in reducing *c-kit* mRNA could be used for the treatment of other cancers resistant to imatinib mesylate, such as GISTs which, although they respond to this drug at first, often develop new mutations that confer them resistance. We have demonstrated here that the exclusive inhibition of the mutant allele of *c-kit* is more efficient than the inhibition of both mutant and wild type alleles and this could be a good strategy for decreasing cancer cell survival. The system could also be used in combination with other therapies for the treatment of *c-kit* related malignancies.

CONCLUSIONS

- RNAi-mediate inhibition is efficient in discriminating between mutant and wild type alleles from c-*kit* oncogene, thus have therapeutic potential; such as in the case of mast cell leukaemia, caused by dominant, gain-of-function mutations.
- RNAi-mediated degradation of mutant c-*kit* triggers a decrease in cell proliferation and induces apoptosis.
- Cell death is induced in both sensitive to imatinib mesylate (HMC1.1) or resistant (HMC1.2) cell lines.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

MCL: Mast cell leukemia
GIST: Gastrointestinal stromal tumours
pac: puromycin-resistance gene
V560G: Val560Gly mutation
D816V: Asp816Val mutation
Tot: Total
Mut: Mutant

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