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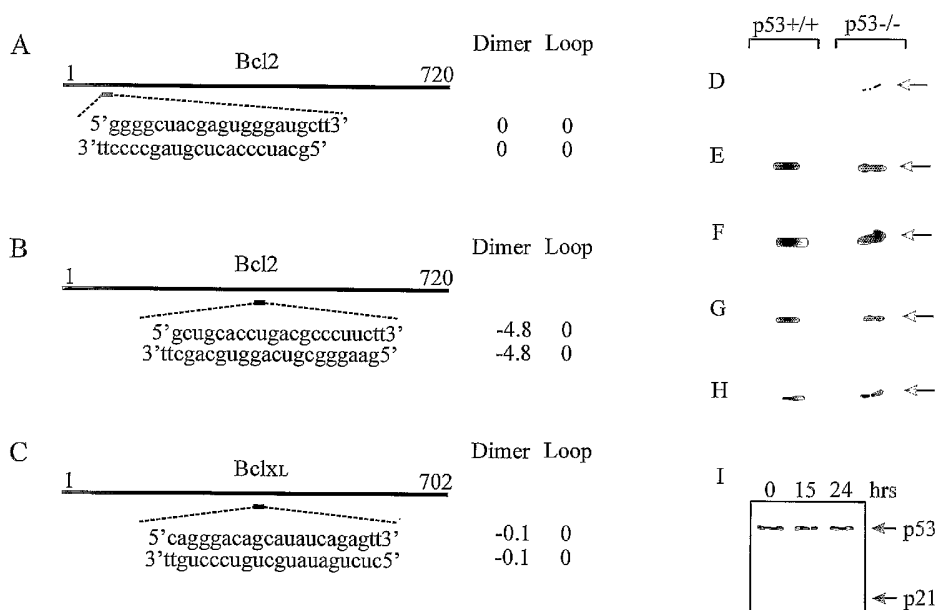
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(54) Title: REGULATION OF GENE EXPRESSION



(57) Abstract: The present invention relates to a method of regulating apoptosis. The method comprises the step of introducing into a cell an RNA construct comprising a nucleotide sequence which is homologous to mRNA within said cell. The mRNA within the cell includes genetic information of a gene element involved in the regulation of apoptosis. The invention also relates to an siRNA construct having a nucleotide sequence which is homologous to mRNA transcribed from a gene element involved in the regulation of apoptosis.

## REGULATION OF GENE EXPRESSION

### FIELD OF THE INVENTION

- 5 This invention relates to the regulation of gene expression and in particular to the use of small interfering RNAs (siRNAs) in the regulation of apoptotic mediators.

### BACKGROUND TO THE INVENTION

- 10 The pathways governing apoptosis in mammalian cells are complex and the pro- and anti-apoptotic permutations regulating cell viability vary according to species, cell type, and also between normal and cancer cells (reviewed in Johnstone et al., 2002; Reed 2002; Cory and Adams, 2002). Imbalance in favour of cell survival enables tumour progression and resistance to anti-cancer drugs. For example, the pro-
- 15 apoptotic *Bax* gene is frequently mutated in DNA mismatch repair-deficient tumours due to an unstable G8 tract at nucleotides 114 – 121 (Ionov et al., 1993; Rampino et al., 1997; Zhang et al., 2000). When both *Bax* alleles are mutated the resultant Bax deficiency confers resistance to non-steroidal anti-inflammatory drugs (NSAIDs) such as sulindac and indomethicin (He et al., 1999; Yamamoto et al, 1999; Zhang et
- 20 al., 2000).

Since predisposition to colorectal cancer is commonly associated with defective mismatch repair (Lynch, 1999), mutation in Bax may explain acquired resistance to sulindac when administered as chemopreventative agent. Indeed, sulindac enables

25 clonal expansion of Bax-deficient cells in culture (Zhang et al., 2000) and may

similarly favour clonal expansion of Bax-deficient cells in the colorectal epithelium of patients with inherited mismatch repair defects. However, Bax-deficient cells remain sensitive to 5-fluorouracil (5-FU) which activates p53-dependent apoptosis (Bunz et al., 1999; Zhang et al., 2000) and is the mainstream therapy for colorectal  
5 cancer.

There is a need to identify cellular pathways and apoptotic mediators that influence the survival of cancer cells. Such cellular pathways and apoptotic mediators represent promising targets for anti-cancer therapies.

10

The current model for mechanism of RNAi is based upon the observation that the introduced dsRNA is bound and cleaved by endonuclease RNase III to generate 21- and 22-nucleotide products. These small interfering RNAs (siRNAs) remain stably complexed with the endonuclease. The resulting dsRNA-protein complexes appear to  
15 represent the active effectors of selective degradation of homologous mRNA (Hamilton & Baulcombe, 1999; Zamore et al., 2000; Elbashir et al., 2001a). Indeed, it has been established that duplexes of 21-nucleotide RNAs are sufficient to suppress expression in endogenous genes in mammalian cells (Elbashir et al., 2001b). This was demonstrated by selective silencing of endogenous lamin A/C  
20 expression in human epithelial cells following introduction of the cognate siRNA duplex. Thus, introduction of siRNA into mammalian cells is sufficient to selectively target homologous mRNA and silence gene expression. Importantly, siRNAs do not induce the non-specific interferon response, observed with dsRNAs  
30 nucleotides long (Minks et al., 1979).

RNA interference is triggered by double-stranded RNA (dsRNA) and enables gene silencing by targeting specific mRNA transcripts for degradation: the silencing process is thus post-transcriptional and host cell genomic integrity is maintained. In mammalian cells RNA interference is induced by short interfering RNA (siRNA) duplexes (Elbashir et al., 2001). SiRNA duplexes target homologous mRNA for degradation with exquisite selectivity and very high, sustained efficacy. Moreover, gene silencing by a single dose of siRNA is achieved within a few days (Elbashir et al, 2001; Jiang and Milner, 2002) and avoids the need for protracted long-term selection procedures such as those necessary to establish gene knock-out cells. Thus RNA interference permits functional dissection of apoptotic pathways by silencing anti-apoptotic genes in cells in which specific pro-apoptotic genes are already deleted.

#### STATEMENTS OF THE INVENTION

According to the present invention there is provided a method of regulating apoptosis, said method comprising introducing into a cell an RNA construct comprising a nucleotide sequence which is homologous to mRNA within said cell, wherein said mRNA includes genetic information of a gene element involved in the regulation of apoptosis.

20

Preferably the degree of homology of said RNA construct nucleotide sequence and said mRNA is at least 50% sequence identity, preferably at least 75%, more preferably at least 85% or at least 95% identity. Most preferably the degree of homology of said RNA construct nucleotide sequence and said mRNA is 92 to 95%,

97 to 99% or 100%. Preferably no more than 4 nucleotides of the RNA construct nucleotide sequence and the corresponding mRNA sequence are non identical. Preferably no more than 3, 2 or 1 nucleotides of the RNA construct nucleotide sequence and the corresponding mRNA sequence are non identical.

5

Preferably said gene element is involved in the suppression of apoptosis.

In a preferred embodiment of the invention said gene element is Bcl-2.

10 Preferably the gene element comprises a nucleic acid molecule, or part thereof, selected from the group consisting of:

(i) a nucleic acid molecule as represented by Figure 6 or a functional fragment thereof;

(ii) a nucleic acid molecule which hybridises to any of the nucleic acid sequences in (i) and which has siRNA activity;

15

(iii) a nucleic acid molecule which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) and/or (ii) above.

In a further preferred embodiment of the invention, said gene element is Bcl-x<sub>L</sub>.

20

Preferably the gene element comprises a nucleic acid molecule, or part thereof, selected from the group consisting of:

(i) a nucleic acid molecule as represented by Figure 7 or a functional fragment thereof;

(ii) a nucleic acid molecule which hybridises to any of the nucleic acid sequences in (i) and which has siRNA activity;

(iii) a nucleic acid molecule which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) and/or (ii) above.

5

In a further preferred embodiment of the invention, said gene element is a viral homologue of a gene involved in the regulation of apoptosis.

The term viral homologue is used herein to refer to either a structural or functional  
10 homologue wherein the viral gene may have a certain degree of structural homology to an anti-apoptotic gene such as Bcl-2 or Bcl-x<sub>L</sub>. Preferably the degree of homology is at least 50% sequence identity, more preferably at least 75% sequence identity, more preferably at least 85% identity and more preferably at least 95% sequence identity. Alternatively or additionally, said homologue may be a functional  
15 homologue of an anti-apoptotic gene wherein said homologue has a role in the regulation, preferably suppression, of cellular apoptosis.

Silencing of Bcl-2 was found to induce massive p53-dependent apoptosis and to occur under normal cell growth conditions (i.e. without recourse to genotoxic drugs  
20 necessary to activate p53 as a transcription factor).

The present invention concerns a novel pro-apoptotic function of p53 under Bcl-2 regulation, thus creating a constitutive Bcl-2/p53 axis regulating apoptosis in human colorectal epithelial cells.

Further experiments using isogenic clones of Bax<sup>+/+</sup> and Bax<sup>-/-</sup> cells and caspase 2 siRNA, clearly demonstrate that both Bax and caspase 2 are essential mediators of the Bcl-2/p53 apoptotic pathway. (Bax <sup>+/+</sup> is a HCT116 derived cell line which has Bax gene, Bax<sup>-/-</sup> is a HCT116 derived cell line in which Bax gene has been knocked  
5 out).

The present invention also provides an siRNA construct having a nucleotide sequence which is homologous to mRNA transcribed from a gene element involved in the regulation of apoptosis.

10

Preferably the siRNA construct is from 15 to 25 nucleotides in length. More preferably the siRNA construct is from 19 to 23 nucleotides in length.

Preferably the siRNA construct comprises;

- 15 (i) a nucleotide sequence that is homologous to a part or fragment of the nucleic acid sequences in Figure 6;
- (ii) a nucleotide sequence which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) above.

20 In a preferred embodiment of the invention said siRNA construct hybridises to the nucleic acid sequence in Figure 6 under stringent hybridisation conditions. (Sambrook et al., (1989) Molecular Cloning; A Laboratory Approach).

Preferably the siRNA construct comprises a nucleotide sequence that is homologous to Bcl-2 mRNA nucleotides 354-372.

In an alternative embodiment of the invention the siRNA construct comprises;

- 5           (i)     a nucleotide sequence that is homologous to a part or fragment of the nucleic acid sequence in Figure 7;
- (ii)     a nucleotide sequence which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) above.

- 10   In a preferred embodiment of the invention said siRNA construct hybridises to the nucleic acid sequence in Figure 7 under stringent hybridisation conditions.

Preferably the siRNA construct comprises a nucleotide sequence that is homologous to Bcl-x<sub>L</sub> nucleotides 347-366.

15

- In a further embodiment the invention also provides a method of treating a disease or condition associated with inappropriate apoptosis comprising administering to a subject an RNA construct wherein said RNA construct has a nucleotide sequence which is homologous to mRNA present within a cell of said subject and wherein said
- 20   mRNA includes genetic information of a gene element involved in the regulation of apoptosis.



The term 'inappropriate apoptosis' is used herein to refer to the situation wherein the level of apoptosis is imbalanced in an undesirable way, for example, towards cell survival, which enables tumour progression and resistance to anti-cancer drugs.

- 5 In another embodiment the invention provides for the use of an RNA construct in the regulation of apoptosis in a cell, wherein said RNA construct has a nucleotide sequence which is homologous to mRNA within the cell and wherein said mRNA includes genetic information of a gene element involved in the regulation of apoptosis.

10

Accordingly the present invention provides an RNA construct for use as a medicament.

- The present invention also provides for the use of an RNA construct for the  
15 manufacture of a medicament for the treatment of a disease or condition associated with inappropriate apoptosis.

The present invention also provides for the use of an RNA construct for the manufacture of a medicament to induce apoptosis.

20

In one embodiment of the invention the disease or condition associated with inappropriate apoptosis is colorectal cancer.

In an alternative embodiment of the invention, the disease or condition associated with inappropriate apoptosis is a viral induced cancer.

In an alternative embodiment of the invention, the disease or condition associated with inappropriate apoptosis is colon cancer, leukaemia, breast cancer, cervical cancer, lung cancer, prostate cancer, lymphomas, liver cancer, renal cell carcinoma, neuroblastoma, adenocarcinoma, pancreatic cancer, malignant melanoma, uterine leiomyomas.

## 10 DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described by way of example only and with reference to the following diagrams;

15 **Figure 1** illustrates the siRNA sequences used, and expression of Bcl-2 in HCT116 cells. **a** and **b**, Bcl-2 siRNA sequences, equivalent to Bcl-2 mRNA nucleotides 77-95 and 354-372 respectively; and **c**, Bcl-x<sub>L</sub> siRNA sequence, nucleotides 347-366 (See WO 03/008573 for methods of RNA production). Predicted secondary structures with propensity for base-pairing out of register (dimers) or for forming stem-loop  
20 structures (loops) were derived using Vector NTI. Anti-sense RNA controls employed Bcl-2 anti-sense nucleotides 354-372, and Bcl-x<sub>L</sub> anti-sense nucleotides 347-366. Control siRNA (Jiang and Milner, 2002) and lamin A/C siRNA (Elbashir et al., 2001) were also used in this study. **d-h**, Immunoblots of Bcl-2 protein (arrowed) in HCT116 p53<sup>+/+</sup> and p53<sup>-/-</sup> cell lysates using different anti-Bcl-2 antibodies: **d**, =

N19, Santa Cruz; **e**, = C-2, Santa Cruz; **f**, = Ab-1, Oncogene; this antibody gave non-specific cross-reactivity with multiple cellular proteins (not shown); **g**, = Ab-2, Oncogene; and **h**, = BD Pharmingen. The C-2 antibody (Santa Cruz) was used in all subsequent experiments. **i**, Immunoblots of p53 and p21 in HCT116 p53+/+ cells at  
 5 different times after transfection with control siRNA as indicated.

**Figure 2** illustrates siRNA silencing of Bcl-2 induces p53-dependent apoptosis.

Isogenic clones of p53+/+ and p53-/- HCT116 cells were cultured and transfected with siRNAs as described previously (Methods). Transfection efficiency = 70 –80%.

10 **a**, Immunoblots of Bcl-2 (closed arrows), and lamin A/C (open arrow). Times post-transfection with control siRNA, Bcl-2 siRNAs and lamin A/C siRNA are as indicated. **b**, Phase contrast images of p53+/+ and p53-/- HCT116 cells at 24 and 48 hr post-transfection with control siRNA, Bcl-2 siRNAs or with lamin A/C siRNA. **c**, Apoptotic cells confirmed by DNA laddering. M = marker; lanes 1 = control siRNA;  
 15 lanes 2 = Bcl-2(a) siRNA; lanes 3 = Bcl-2(b) si-RNA. Cells were harvested for analysis 48 hr post-transfection. **d**, Annexin V-positive apoptotic cells detected by FACS analysis (Methods). Cells were harvested at 24 and 48 hr post-transfection as indicated. □ = control siRNA; ▨ = Bcl-2(a) siRNA; ■ = Bcl-2(b) siRNA; ▩ = Bcl-2(b) anti-sense RNA. In all subsequent experiments Bcl-2(b) siRNA was employed  
 20 to silence Bcl-2 expression, and apoptosis was confirmed by the independent techniques of DNA laddering and annexin V labelling with FACS analysis. **e**, Cytochrome c distribution in cells at the time of transfection (0 hr) and release into the cytosol of non-adherent cells collected 48 hr following transfection with Bcl-2 siRNA in HCT116 p53+/+ cells. P = pellet fraction; C = cytosolic fraction.

**Figure 3.**

p53-independent apoptotic pathways in isogenic clones of HCT116 cells.

**a – c**, siRNA silencing of the anti-apoptotic gene Bcl-x<sub>L</sub> using the siRNA sequence shown in Fig. 1c. **a**, Immunoblot of Bcl-x<sub>L</sub> protein at different times after transfection with control siRNA or Bcl-x<sub>L</sub> siRNA. **b**, Phase contrast images of cells at 48 and 72 hr post-transfection with control siRNA or Bcl-x<sub>L</sub> siRNA. **c**, Early apoptotic cells detected by annexin V labelling and FACS analysis. Cells were harvested for analysis at 48 and 72 hr as indicated. □ = control siRNA; ■ = Bcl-x<sub>L</sub> siRNA; ▨ = Bcl-x<sub>L</sub> anti-sense RNA. **d**, Apoptosis induced by treatment with sulindac (Methods). Phase contrast images of cells at 24 and 48 hr post-treatment with sulindac, which activates Bax-dependent, p53-independent apoptosis (Zhang et al., 2000). Apoptosis was confirmed by DNA laddering and by FACS analysis of cells labelled with annexin V (not shown).



**Figure 4.**

Apoptosis following silencing of Bcl-2 or Bcl-x<sub>L</sub> depends upon Bax and caspase 2.

**a**, Phase contrast images of isogenic clones of Bax<sup>+/+</sup> and Bax<sup>-/-</sup> HCT116 cells at 72 hr post-transfection with control siRNA, with Bcl-2 siRNA or with Bcl-x<sub>L</sub> siRNA, as indicated. **b**, Apoptotic cells in isogenic clones of HCT116 cells detected by labelling with annexin V and FACS analysis. The cells were harvested at 72 hr post-transfection with control siRNA, Bcl-2 siRNA or Bcl-x<sub>L</sub> siRNA as indicated; □ = Bax<sup>+/+</sup> cells and ■ = Bax<sup>-/-</sup> cells. **c**, Apoptotic cells in HCT116 p53<sup>+/+</sup> cells (the same clone as used in Figs. 2 and 3) 72 hr following transfection with caspase 2 siRNA in combination with either Bcl-2 siRNA or Bcl-x<sub>L</sub> siRNA as indicated.

**Figure 5.**

Apoptosis correlates with p53 status in individual human colorectal carcinoma cell lines following silencing of Bcl-2 expression.

- 5 Cells were transfected with Bcl-2 siRNA and apoptotic cells were determined after 48 hr (as described in Fig. 2 legend and methods).  = cell lines expressing endogenous wild -type p53;  = p53-deficient cell lines.

**Figure 6a and 6b** illustrate the Bcl-2 alpha and Bcl-2 beta mRNA and protein  
10 sequence.

**Figure 7** illustrates the Bcl-x<sub>L</sub> mRNA sequence

**Selective silencing of Bcl-2 expression.**

- 15 Paired isogenic clones of HCT116 p53<sup>+/+</sup> and p53<sup>-/-</sup> cells (Bunz et al., 1999; Zhang et al., 2000) were used. To silence Bcl-2 expression we selected two Bcl-2 mRNA target sequences (Figure 1a & b). Both are 100% conserved between human and murine Bcl-2. Silencing of Bcl-2 expression was monitored by immunoblotting the Bcl-2 protein. It should be noted that a previous, well controlled study failed to  
20 clearly detect Bcl-2 in immunoblots of HCT116 cell lysates (Zhang et al., 2000). This observation was confirmed when using the same antibody (N19, Santa Cruz; Figure 1d). However, other antibodies clearly detect Bcl-2 in the HCT116 cells and, importantly, show that Bcl-2 protein levels are equivalent in the p53<sup>+/+</sup> and p53<sup>-/-</sup> cells (Figure 1e-h). The inevitable stress associated with the transfection process was

not sufficient to activate p53 as a transcription factor in p53<sup>+/+</sup> cells as evident from the absence of up-regulation of p21, a p53 target protein (Fig. 1i). The Bcl-2 protein fell to barely detectable levels within 24h transfection with Bcl-2 siRNA (Figure 2a). Interestingly, only one of the two Bcl-2 siRNAs silenced Bcl-2 expression (Bcl-2(b);  
5 Figure 2) indicating that the mRNA sequence homologous to the non-effective siRNA (nucleotides 77 – 95; Fig. 1a) must somehow be protected from recognition and/or degradation by RNA interference. Such protection may arise due to localised mRNA secondary structure or protein-mRNA interactions (see Jiang and Milner, 2002). Control transfections included a random siRNA sequence (control siRNA,  
10 Jiang and Milner, 2002) and lamin A/C siRNA previously shown to suppress lamin A/C protein expression without inducing apoptosis (Elbashir et al., 2001).

#### **Bcl-2 silencing induces p53-dependent apoptosis**

By 48 h massive apoptosis was observed in the p53<sup>+/+</sup> cells transfected with Bcl-2  
15 siRNA (Bcl-2(b); Fig 2 b – d). Apoptosis in cells transfected with Bcl-2 anti-sense RNA (anti-sense sequence 354-372) was negligible (Fig 2d) and equivalent to that observed for control siRNA. This confirms that apoptosis induced by Bcl-2 siRNA in p53<sup>+/+</sup> cells is due to RNA interference. siRNA silencing of lamin A/C failed to induce apoptosis in either the p53<sup>+/+</sup> or p53<sup>-/-</sup> cells (Fig 2b). This demonstrates that  
20 the process of RNA interference *per se* is not sufficient to activate apoptosis in HCT116 p53<sup>+/+</sup> cells. Unexpectedly the p53<sup>-/-</sup> cells failed to undergo apoptosis following silencing of Bcl-2 expression (Fig. 2 b-d). Thus we conclude that selective silencing of Bcl-2 expression induces massive apoptosis of HCT116 colorectal cancer cells and that this effect is dependent upon p53.

It has recently been demonstrated that Bcl-2 can regulate an apoptotic pathway that is activated independently of mitochondrial cytochrome c release and Apaf-1/caspase 9 activation (Marsden et al., 2002). This raises the possibility that p53 may enable this  
5 cytochrome c-independent pathway, thus accounting for the observed differences in apoptosis between p53<sup>+/+</sup> and p53<sup>-/-</sup> cells following silencing of Bcl-2 (Fig. 2). However, analysis of cytochrome c distribution clearly demonstrates release of cytochrome c into the cytosol in p53<sup>+/+</sup> cells undergoing apoptosis following treatment with Bcl-2 siRNA (Fig. 2e). In long exposures of the immunoblots  
10 mitochondrial cytochrome c release was also evident in adherent p53<sup>+/+</sup> cells treated with Bcl-2 siRNA whereas no cytosolic cytochrome c was detectable in parallel cultures of p53<sup>-/-</sup> cells treated with Bcl-2 siRNA (not shown). These results indicate that Bcl-2 silencing induces p53-dependent apoptosis via pathway(s) that involve the release of cytochrome c from the mitochondria.

15

**Bcl-x<sub>L</sub> silencing induces p53-independent apoptosis.**

The integrity of p53-independent apoptotic pathways was next confirmed by silencing the Bcl-x<sub>L</sub> gene, again using RNA interference. Bcl-x<sub>L</sub> is an anti-apoptotic gene (Boise et al., 1993) and in colorectal epithelial cells a decrease in the ratio of  
20 Bcl-x<sub>L</sub>:Bax is sufficient to induce apoptosis (Zhang et al., 2000). Therefore we predicted that selective silencing of Bcl-x<sub>L</sub> should induce apoptotic cell death in both p53<sup>+/+</sup> and p53<sup>-/-</sup> cells. Indeed this proved to be the case. First we ascertained that the selected Bcl-x<sub>L</sub> siRNA sequence (see Fig 1c) effectively reduces Bcl-x<sub>L</sub> protein expression (Figure 3a), and then demonstrated its capacity to induce apoptosis

(Figure 3b-c). Bcl-x<sub>L</sub> protein levels declined between 24 and 48 hr post-transfection with Bcl-x<sub>L</sub> siRNA and subsequently apoptosis was observed in both the p53<sup>+/+</sup> and p53<sup>-/-</sup> cells. This demonstrates that p53 is not required for Bcl-x<sub>L</sub>-regulated apoptotic pathway(s) in colorectal epithelial cells. Further verification of p53-independent  
5 apoptotic pathways was obtained by treating the cells with sulindac which is known to activate Bax-dependent apoptosis (Zhang et al., 2001). Sulindac induced apoptosis in both p53<sup>+/+</sup> and p53<sup>-/-</sup> cells (Figure 3d; see also Zhang et al., 2000). On the basis of these overall results we conclude that the observed lack of apoptosis in p53<sup>-/-</sup> cells treated with Bcl-2 siRNA (Fig. 2) cannot be attributed to either loss of Bax or other  
10 apoptotic pathway suppressed by Bcl-x<sub>L</sub>. This is consistent with the isogenic nature of the two cell clones and indicates that p53 is a selective requirement for apoptosis induced by Bcl-2 silencing.

**Bax and caspase 2 are required for apoptosis following silencing of Bcl-2 or Bcl-**  
15 **x<sub>L</sub>.**

Thus far our results indicate that Bcl-2 constitutively suppresses apoptosis in colorectal cancer cells grown in culture and that, following silencing of Bcl-2 expression, the process of apoptosis requires p53. This is novel and places a pro-apoptotic function of p53 under Bcl-2 regulation. Moreover, this pro-apoptotic  
20 function of p53 does not require treatment of cells with cytotoxic agents such as 5-FU. (Note that the process of RNA interference by itself is not sufficient to activate p53-induced apoptosis, as demonstrated by lack of apoptosis in p53<sup>+/+</sup> cells treated with lamin A/C siRNA, see above). In addition, we show that silencing of Bcl-x<sub>L</sub> induces apoptosis in a p53-independent manner (Fig 3). This is consistent with



previous work identifying Bax as a major player in the apoptotic response of colorectal cancer cells, (Ionov et al., 2000; Zhang et al., 2001; LeBlanc et al., 2002) and Bcl-x<sub>L</sub> as its anti-apoptotic counterpart (when expressed exogenously, Zhang et al., 2001). These combined observations led us to reason that Bcl-2/p53 and Bcl-x<sub>L</sub>/Bax might represent functional partners governing apoptosis in human colorectal epithelial cells. Within this scenario at least two putative apoptotic pathways might be envisaged: (i) Bcl-2/p53 may define an apoptotic pathway that is essentially independent of Bcl-x<sub>L</sub>/Bax; or (ii) Bcl-2/p53 and Bcl-x<sub>L</sub>/Bax may govern inter-related transitions in the apoptotic process. To discriminate between these two alternatives we silenced, individually, Bcl-2 and Bcl-x<sub>L</sub> expression in isogenic clones of Bax<sup>+/+</sup> and Bax<sup>-/-</sup> HCT116 cells (note that the apoptotic response of Bax <sup>+/+</sup> cells is equivalent to Bax<sup>+/+</sup> cells; Zhang et al., 2000). siRNA silencing of Bcl-2 and of Bcl-x<sub>L</sub> induced massive apoptosis in Bax <sup>+/+</sup> cells but failed to induce significant apoptosis in Bax<sup>-/-</sup> cells (Figure 4c and d). This clearly demonstrates that Bax is required for apoptosis in both Bcl-2-regulated and Bcl-x<sub>L</sub>-regulated pathways.

The above results demonstrate that in colorectal carcinoma cells the Bcl-2 and Bcl-x<sub>L</sub> cell death pathways share commonalities in their requirement for Bax, but differ in their requirements for p53. It is possible that p53 is required to prime a pro-apoptotic pathway which is selectively suppressed by Bcl-2, thus lowering the apoptotic threshold consequent to Bcl-2 silencing. To further dissect the functional links between Bcl-2, Bcl-x<sub>L</sub>, p53 and Bax we next asked if caspase 2 is also involved. Apoptosis induced by Bcl-2 or by Bcl-x<sub>L</sub> silencing was blocked when caspase 2 siRNA was co-transfected with either Bcl-2 siRNA or Bcl-x<sub>L</sub> siRNA respectively

(Fig. 4c; see Lassus et al, 2002 for caspase 2 siRNA sequence). siRNA silencing of caspase 2 alone (Fig. 4c), or transfection with anti-sense caspase 2 RNA (not shown), had no apparent effect on cell viability. Overall these results demonstrate that both Bax and caspase 2 are required for apoptosis following silencing of either Bcl-2 or  
5 Bcl-x<sub>L</sub> in p53<sup>+/+</sup> colorectal cancer cells. This is consistent with recent evidence that caspase 2 enables translocation of Bax into the mitochondria and subsequent mitochondrial membrane permeabilisation marked by release of cytochrome c (Marsden et al., 2002).

10 **Effects of Bcl-2 siRNA on individual colorectal carcinoma cell lines of varying p53 status.**

The above experiments involve isogenic clones of HCT116 cells and are thus tightly controlled for genetic variation. To establish the generality of our observations we silenced Bcl-2 in other human colorectal carcinoma cell lines, also defective for  
15 DNA mismatch repair and with defined p53 status (see Methods). In each case the presence of wild type p53 correlated with induction of apoptosis detectable 48 hours post-transfection with Bcl-2 siRNA, whereas p53-deficiency correlated with background levels of apoptosis (Fig. 5). These results confirm our observations with isogenic clones of p53<sup>+/+</sup> and p53<sup>-/-</sup> HCT116 cells and are consistent with concept  
20 that Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells.

## MATERIALS AND METHODS

### Cell lines and transfections

HCT116 clones were cultured in DMEM with 10% FCS. All the cell clones were  
5 cultured with penicillin 100 units ml<sup>-1</sup> and streptomycin 100 µg ml<sup>-1</sup> at 37°C in 5%  
CO<sub>2</sub> in air. Other human colorectal cancer cells lines, also defective for DNA  
mismatch repair (see Branch et al., 1995), were:- LoVo and RKO (p53 wild type);  
DLD1, LS174T, SW48 and HT29 (all p53 mutant). For transfection the cells were  
trypsinised and sub-cultured into 6 well plates (10 cm<sup>2</sup>) without antibiotics, 1.5 x 10<sup>5</sup>  
10 cells per well. Selected 21-nucleotide RNAs were synthesised and HPLC purified  
(MWG; Germany) and annealed into siRNA duplexes according to the instructions  
supplied. 24 h after sub-culture the cells were transfected with siRNA formulated  
into liposomes (Oligofectamine, Life Technologies) according to the manufacturer's  
instructions. The protocol includes a short incubation in serum-free medium but  
15 controls demonstrated that this was not sufficient to activate a p53 response (see  
Results section). siRNA concentration was 0.58 µg per 1.5 X 10<sup>5</sup> cells per well. The  
final volume of culture medium was 1.5 ml per well. Cells were harvested for  
analysis at various times thereafter as indicated in the results. Each experiment with  
HCT116 cells was carried out four or more times. Transfection efficiencies were  
20 established by transfecting with liposomes containing FITC-dextran (Jiang and  
Milner, 2002). Anti-sense RNA controls were included in each experiment using the  
respective anti-sense sequences for Bcl-2(b), Bcl-x<sub>L</sub> and caspase 2 (see Figure 1a.  
and text).

### **Immunoblotting and mitochondrial cytochrome c release**

For immunoblotting the transfected cells were trypsinised, washed in PBS and an aliquot removed for cell counting. The remaining cells were lysed in 50µl lysis buffer (150mM NaCl; 0.5% NP40; 50mM Tris pH 8.0) on ice for 30 min. Samples were diluted 1:1 in 4x strength Laemmli's buffer. Proteins were resolved by 15% SDS-PAGE and electroblotted onto nitrocellulose membrane for antibody detection. Molecular weight markers and purified recombinant human p53 were included as markers (not shown). The following antibodies were employed: for Bcl-2 = N19 and C-2 (Sant Cruz); Ab-1 and Ab-2 (Oncogene; note that Ab-1 gave non-specific background with multiple cellular proteins, not shown); and BD (Pharmingen) (Figure 1b). The C-2 antibody gave the cleanest results and was subsequently used throughout this work. Lamin A/C = antibody 636; Santa Cruz); Bcl-x<sub>L</sub> = Bcl-X antibody (Pharmingen; this antibody gave a relatively high non-specific background). P53 = DO-1 antibody (Oncogene) and caspase 2 = caspase-2L antibody (F-7; Santa Cruz). Visualisation of bound antibodies was by enhanced chemiluminescence (Roche). Cell fractionations and cytochrome c determinations were carried out as described in Marsden et al (2002, supplementary information).

### **Cell growth, cell cycle analysis and apoptosis**

Cell growth curves were determined by cell counting. Induction of apoptosis by sulindac (see Figure 3) employed sulindac sulphide 120µM (Calbiochem). For cell cycle analysis the cells were harvested, washed with PBS and fixed in 90% ethanol overnight at -20°C. The fixed cells were pelleted, washed in PBS and resuspended in

PBS containing 0.1 µg/ml propidium iodide with 200 U/ml RNase A and analysed by FACS. Apoptotic cells were identified using annexin-V-Fluos (Boehringer) following the manufacturer's protocol. Apoptosis was also verified by DNA laddering using the Suicide-track DNA ladder isolation kit (Oncogene) according to  
5 the manufacturer's instructions.

### Discussion

In the present study we have used isogenic cell clones and siRNA to obtain defined combinations of pro- and anti-apoptotic gene expression in cells that are otherwise  
10 genetically equivalent. Our observations indicate a new cell death pathway in which Bcl-2 constitutively suppresses p53-dependent apoptosis. Apoptosis can also be induced by treating the cells with agents such as 5'FU to activate p53 (Zhang et al., 2000 and results not shown). This is consistent with established evidence that activated p53 functions up-stream of Bcl-2 in response to genotoxic stress (see  
15 Strasser et al., 1994; and reviews by Johnstone et al, 2002; Cory and Adams 2002). To accommodate our present observations within the context of previous studies we suggest that Bcl-2 constitutively suppresses a novel pro-apoptotic function of p53 and that exposure to genotoxic stress over-rides Bcl-2 suppression by inducing the transactivation potential of p53. Once activated as a transcription factor p53 has the  
20 capacity to alter the expression ratios of Bcl-2 and Bcl-x<sub>L</sub> (down-regulated) and Bax (up-regulated) in favour of apoptosis (see Johnstone et al., 2002). From a clinical point of view this has proved very useful for anti-cancer therapy but carries the inherent risk of non-specific cytotoxicity and genotoxicity caused by p53-activating agents.

The present invention shows that p53 possesses pro-apoptotic properties that appear to be constitutively active, albeit suppressed by Bcl-2. Bcl-2 is identified as a potential and promising target for anti-cancer therapy for colorectal cancer (see also  
5 Reed et al., 2002) and the accessibility of Bcl-2 for siRNA silencing is demonstrated. The survival of other epithelial tumours may be similarly susceptible to Bcl-2 silencing. With the development of RNA interference the selective silencing of specific genes is now a realistic possibility, and the continual inventive evolution of targeted delivery systems (see, for example, Hood et al., 2002) should enable  
10 application of RNA interference to prevent and to treat cancer.

The present invention also carries important implications for patients with inherited DNA mismatch repair deficiencies and associated pre-disposition to colorectal cancer. In particular it argues against the use of sulindac as chemo-preventative in  
15 such patients since it is well established that defective mismatch repair renders the Bax gene susceptible to mutation and favours clonal expansion of Bax-deficient cells. In the present study we demonstrate that Bax is an essential mediator of apoptosis regulated by the newly discovered Bcl-2/p53 pathway (see above). It follows that, in patients with mismatch repair defects, any selective pressure for Bax-  
20 deficient cells may exacerbate tumorigenesis and should be avoided.

The constitutive pro-apoptotic function of p53 may be linked with apical apoptosis in the normal colorectal epithelium. If so, failure of apoptosis in colorectal epithelial tumours might reflect inappropriate suppression of intrinsic p53-induced apoptosis.

A strong candidate in this regard is Bcl-2 which constitutively blocks p53-induced apoptosis and enables the survival of colorectal cancer cells. Such a model is consistent with the late onset of p53 mutation in the malignant progression of colorectal cancer. It also re-enforces Bcl-2 as a prime target for development of novel  
5 anti-cancer agents.

Viral homologues of cellular anti-apoptotic genes such as Bcl-2 represent promising targets for the treatment of viral-induced cancers. The silencing of human Bcl-2 expression identifies a Bcl-2 mRNA sequence that is accessible for the RNAi  
10 machinery. Only one of two different anti-Bcl-2 siRNAs has been found to be effective, underscoring the importance of target sequence selection and verification during the development of any anti-viral therapy based upon RNA interference. Viral homologues of Bcl-2 (v-Bcl-2) containing viral-specific nucleotide sequences accessible for RNAi will represent promising targets for RNAi-based anti-viral/anti-  
15 cancer therapies.

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## CLAIMS

1. A method of regulating apoptosis, said method comprising introducing into a cell an RNA construct comprising a nucleotide sequence which is homologous to mRNA within said cell, wherein said mRNA includes genetic information of a gene element involved in the regulation of apoptosis.
2. A method according to claim 1 wherein said gene element is involved in the suppression of apoptosis.
3. A method according to claim 1 or claim 2 wherein said gene element is Bcl-2.
4. A method according to claim 3 wherein the gene element comprises a nucleic acid molecule, or part thereof, selected from the group consisting of:
- (i) a nucleic acid molecule as represented by Figure 6 or a functional fragment thereof;
  - (ii) a nucleic acid molecule which hybridises to any of the nucleic acid sequences in (i) and which has siRNA activity;
  - (iii) a nucleic acid molecule which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) and/or (ii) above.
5. A method according to claim 1 or claim 2 wherein said gene element is Bcl-X<sub>L</sub>.

6. A method according to claim 5 wherein the gene element comprises a nucleic acid molecule, or part thereof, selected from the group consisting of:
- (i) a nucleic acid molecule as represented by Figure 7 or a functional fragment thereof;
  - 5 (ii) a nucleic acid molecule which hybridises to any of the nucleic acid sequences in (i) and which has siRNA activity;
  - (iii) a nucleic acid molecule which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) and/or (ii) above.
- 10 7. A method according to claim 1 or claim 2 wherein said gene element is a viral homologue of a gene involved in the regulation of apoptosis.
8. An siRNA construct having a nucleotide sequence which is homologous to mRNA transcribed from a gene element involved in the regulation of apoptosis.
- 15 9. An siRNA construct according to claim 8 wherein said construct is from 15 to 25 nucleotides in length.
10. An siRNA construct according to claim 9 wherein said construct is from 19 to 20 23 nucleotides in length.
11. An siRNA construct of any of claims 8 to 10 comprising;
- (i) a nucleotide sequence that is homologous to a part or fragment of the nucleic acid sequences in Figure 6;

(ii) a nucleotide sequence which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) above.

12. An siRNA construct according to any of claims 8 to 11 comprising a  
5 nucleotide sequence that is homologous to Bcl-2 mRNA nucleotides 354-372.

13. An siRNA according to any of claims 8 to 10 comprising;

(i) a nucleotide sequence that is homologous to a part or fragment of the nucleic acid sequence in Figure 7;

10 (ii) a nucleotide sequence which is degenerate as a result of the genetic code to the nucleic acid sequence of (i) above.

14. An siRNA construct according to any of claims 8 to 10 or claim 13 comprising a nucleotide sequence that is homologous to Bcl-x<sub>L</sub> nucleotides 347-366.

15

15. A method of treating a disease or condition associated with inappropriate apoptosis comprising administering to a subject an RNA construct wherein said RNA construct has a nucleotide sequence which is homologous to mRNA present within a cell of said subject and wherein said mRNA includes genetic information of a gene  
20 element involved in the regulation of apoptosis.

16. Use of an RNA construct of any of claims 8 to 14 in the regulation of apoptosis in a cell, wherein said RNA construct has a nucleotide sequence which is

homologous to mRNA within the cell and wherein said mRNA includes genetic information of a gene element involved in the regulation of apoptosis.

17. An RNA construct for use as a medicament.

5

18. Use of an RNA construct for the manufacture of a medicament to induce apoptosis.

19. Use of an RNA construct for the manufacture of a medicament for the  
10 treatment of colorectal cancer.

20. Use of an RNA construct for the manufacture of a medicament for the treatment of viral induced cancer.

15

20

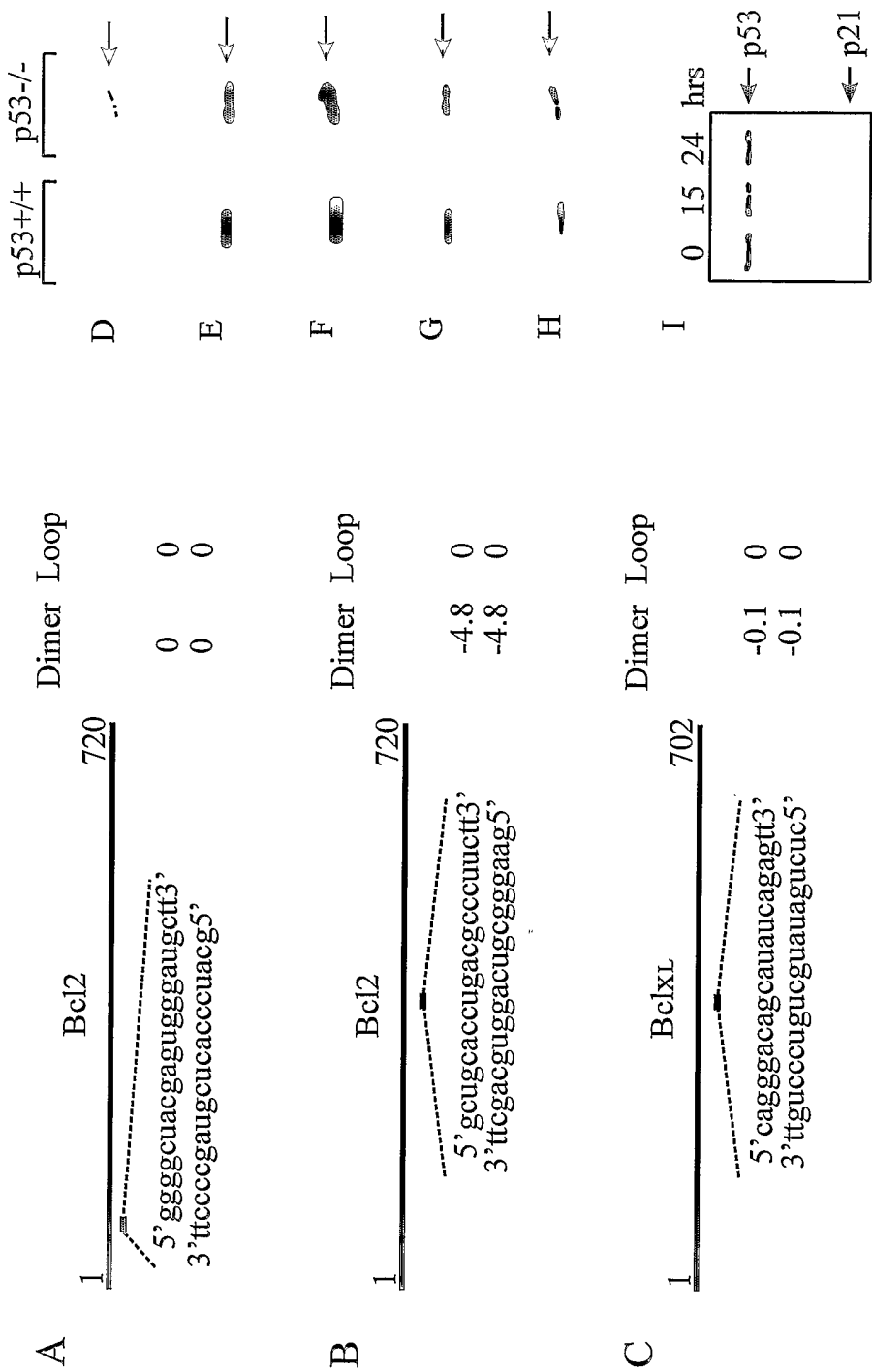


Fig. 1

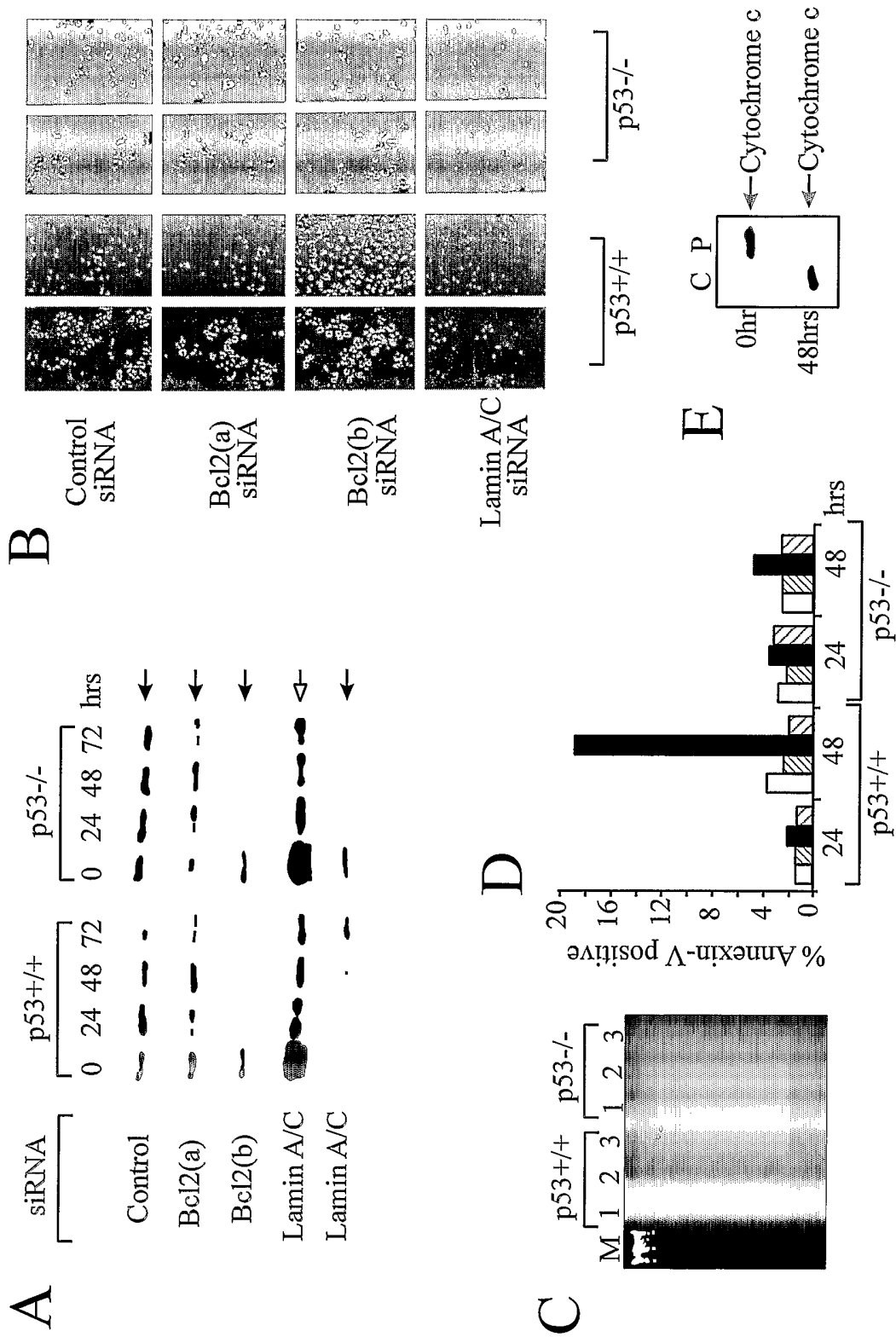


Fig. 2



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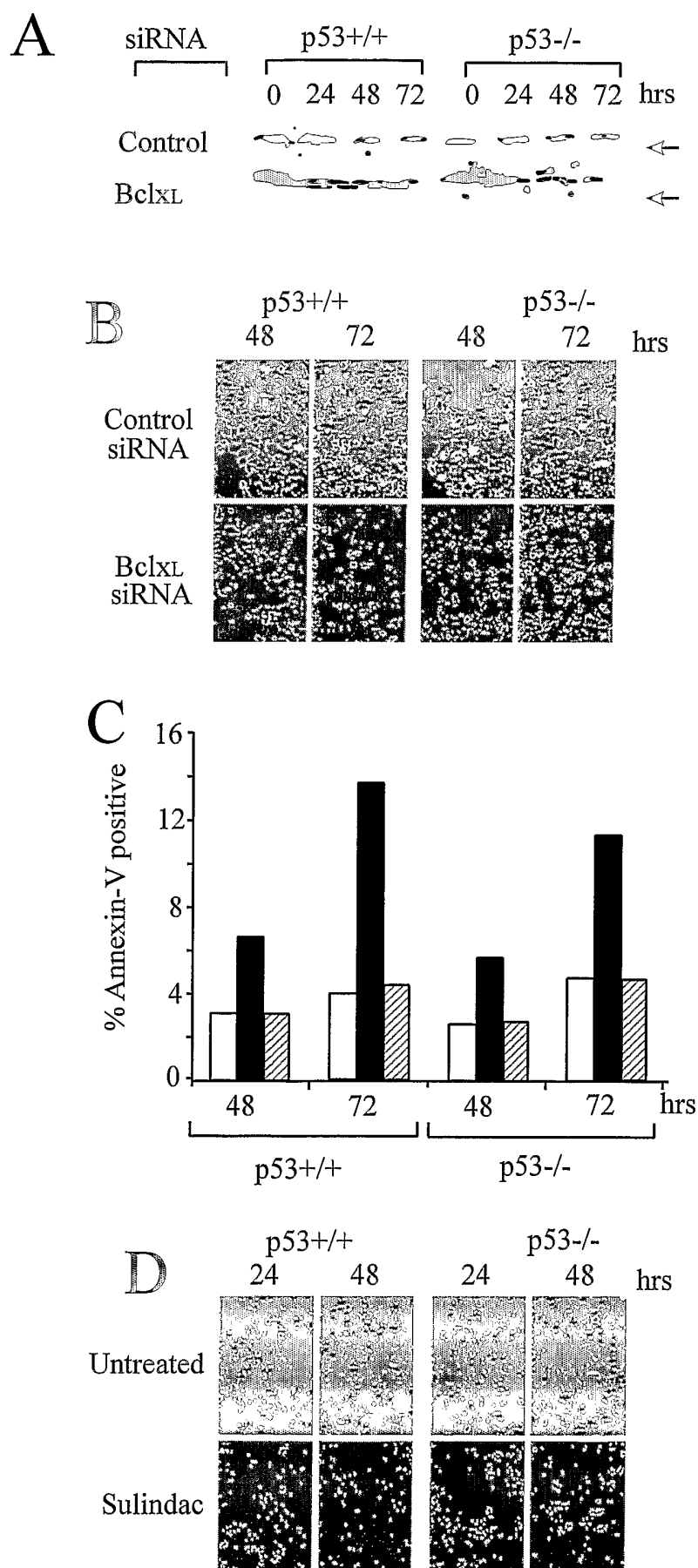
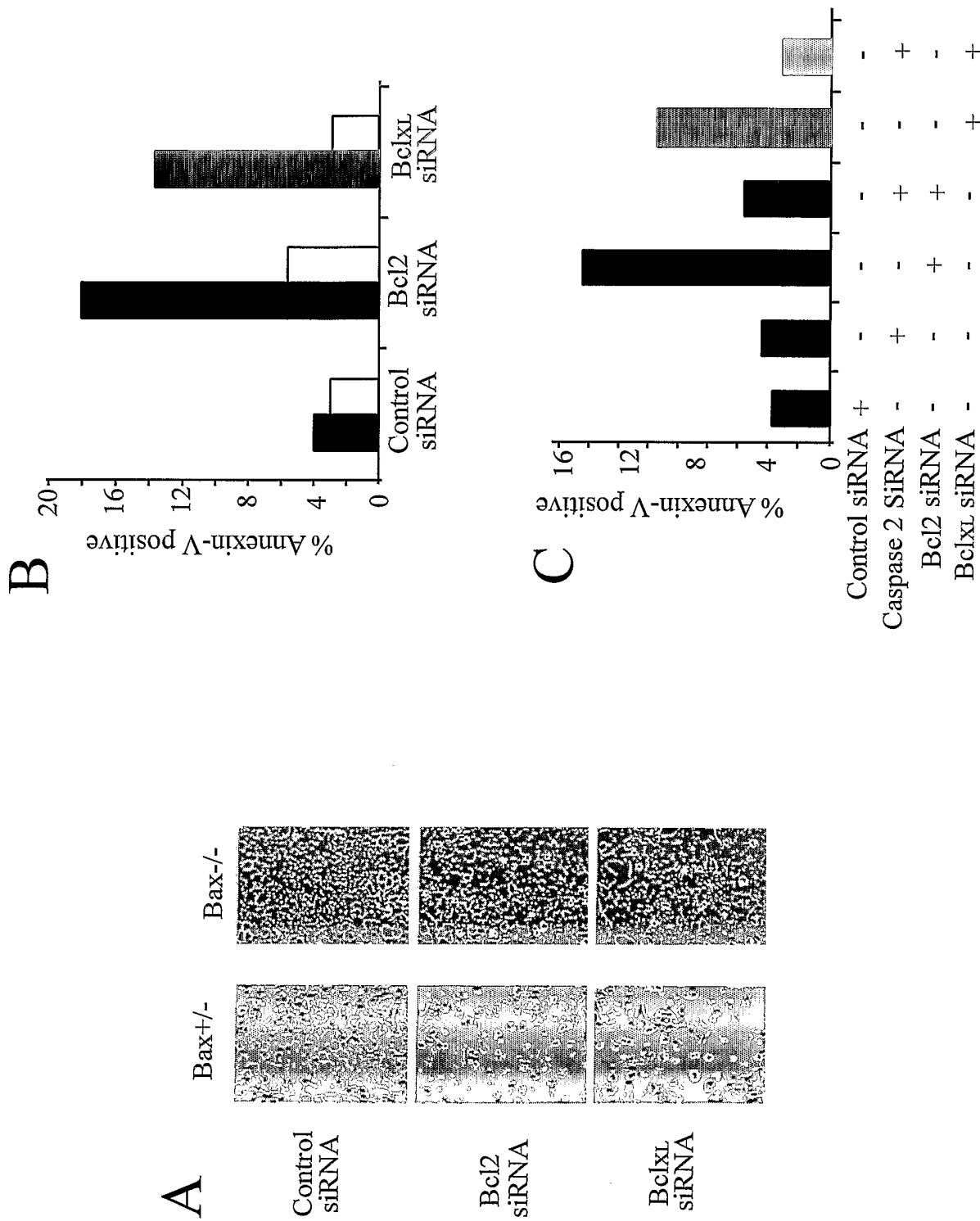


Fig. 3

Fig. 4



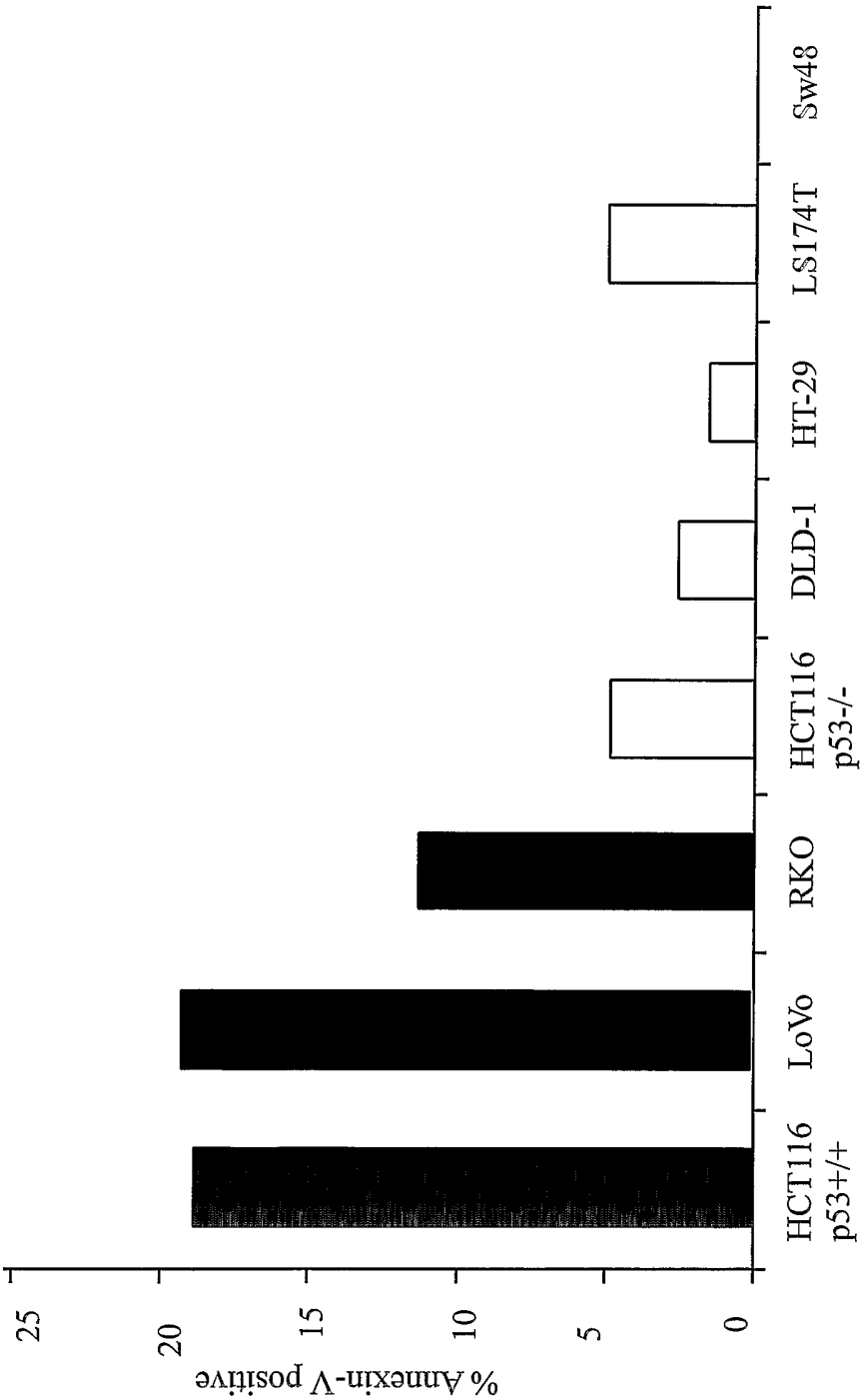


Fig. 5

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Figure 6a: Bcl2 alpha (720bps) (GenBank NM\_000633)

```

M  A  H  A  G  R  T  G  Y  D  N  R  E  I  V  M  K  Y  I  H
atg gcg cac gct ggg aga aca ggg tac gat aac cgg gag ata gtg atg aag tac atc cat

Y  K  L  S  Q  R  G  Y  E  W  D  A  G  D  V  G  A  A  P  P
tat aag ctg tcg cag agg ggc tac gag tgg gat gcg gga gat gtg ggc gcc gcg ccc ccg

G  A  A  P  A  P  G  I  F  S  S  Q  P  G  H  T  P  H  T  A
ggg gcc gcc ccc gcg ccg ggc atc ttc tcc tcg cag ccc ggg cac acg ccc cat aca gcc

A  S  R  D  P  V  A  R  T  S  P  L  Q  T  P  A  A  P  G  A
gca tcc cgg gac ccg gtc gcc agg acc tcg ccg ctg cag acc ccg gct gcc ccc ggc gcc

A  A  G  P  A  L  S  P  V  P  P  V  V  H  L  T  L  R  Q  A
gcc gcg ggg cct gcg ctc agc ccg gtg cca cct gtg gtc cac ctg acc ctc cgc cag gcc

G  D  D  F  S  R  R  Y  R  R  D  F  A  E  M  S  R  Q  L  H
ggc gac gac ttc tcc cgc cgc tac cgc cgc gac ttc gcc gag atg tcc agg cag ctg cac

L  T  P  F  T  A  R  G  R  F  A  T  V  V  E  E  L  F  R  D
ctg acg ccc ttc acc gcg cgg gga cgc ttt gcc acg gtg gtg gag gag ctc ttc agg gac

G  V  N  W  G  R  I  V  A  F  F  E  F  G  G  V  M  C  V  E
ggg gtg aac tgg ggg agg att gtg gcc ttc ttt gag ttc ggt ggg gtc atg tgt gtg gag

S  V  N  R  E  M  S  P  L  V  D  N  I  A  L  W  M  T  E  Y
agc gtc aac cgg gag atg tcg ccc ctg gtg gac aac atc gcc ctg tgg atg act gag tac

L  N  R  H  L  H  T  W  I  Q  D  N  G  G  W  D  A  F  V  E
ctg aac cgg cac ctg cac acc tgg atc cag gat aac gga ggc tgg gat gcc ttt gtg gaa

L  Y  G  P  S  M  R  P  L  F  D  F  S  W  L  S  L  K  T  L
ctg tac ggc ccc agc atg cgg cct ctg ttt gat ttc tcc tgg ctg tct ctg aag act ctg

L  S  L  A  L  V  G  A  C  I  T  L  G  A  Y  L  G  H  K  *
ctc agt ttg gcc ctg gtg gga gct tgc atc acc ctg ggt gcc tat ctg ggc cac aag tga

```

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Figure 6b: Bcl2 beta(618bps) (GenBank NM\_000657)

```

M  A  H  A  G  R  T  G  Y  D  N  R  E  I  V  M  K  Y  I  H
ATG GCG CAC GCT GGG AGA ACG GGG TAC GAC AAC CGG GAG ATA GTG ATG AAG TAC ATC CAT

Y  K  L  S  Q  R  G  Y  E  W  D  A  G  D  V  G  A  A  P  P
TAT AAG CTG TCG CAG AGG GGC TAC GAG TGG GAT GCG GGA GAT GTG GGC GCC GCG CCC CCG

G  A  A  P  A  P  G  I  F  S  S  Q  P  G  H  T  P  H  P  A
GGG GCC GCC CCC GCA CCG GGC ATC TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT CCA GCC

A  S  R  D  P  V  A  R  T  S  P  L  Q  T  P  A  A  P  G  A
GCA TCC CGC GAC CCG GTC GCC AGG ACC TCG CCG CTG CAG ACC CCG GCT GCC CCC GGC GCC

A  A  G  P  A  L  S  P  V  P  P  V  V  H  L  A  L  R  Q  A
GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT GTG GTC CAC CTG GCC CTC CGC CAA GCC

G  D  D  F  S  R  R  Y  R  G  D  F  A  E  M  S  S  Q  L  H
GGC GAC GAC TTC TCC CGC CGC TAC CGC GGC GAC TTC GCC GAG ATG TCC AGC CAG CTG CAC

L  T  P  F  T  A  R  G  R  F  A  T  V  V  E  E  L  F  R  D
CTG ACG CCC TTC ACC GCG CGG GGA CGC TTT GCC ACG GTG GTG GAG GAG CTC TTC AGG GAC

G  V  N  W  G  R  I  V  A  F  F  E  F  G  G  V  M  C  V  E
GGG GTG AAC TGG GGG AGG ATT GTG GCC TTC TTT GAG TTC GGT GGG GTC ATG TGT GTG GAG

S  V  N  R  E  M  S  P  L  V  D  N  I  A  L  W  M  T  E  Y
AGC GTC AAC CGG GAG ATG TCG CCC CTG GTG GAC AAC ATC GCC CTG TGG ATG ACT GAG TAC

L  N  R  H  L  H  T  W  I  Q  D  N  G  G  W  V  G  A  S  G
CTG AAC CGG CAC CTG CAC ACC TGG ATC CAG GAT AAC GGA GGC TGG GTA GGT GCA TCT GGT

D  V  S  L  G  *
GAT GTG AGT CTG GGC TGA

```

**Figure 7**

Human Bcl-xL mRNA (NM\_138578)

atg tct cag agc aac cgg gag ctg gtg gtt gac ttt ctc tcc tac aag ctt tcc cag aaa  
gga tac agc tgg agt cag ttt agt gat gtg gaa gag aac agg act gag gcc cca gaa ggg  
act gaa tcg gag atg gag acc ccc agt gcc atc aat ggc aac cca tcc tgg cac ctg gca  
gac agc ccc gcg gtg aat gga gcc act ggc cac agc agc agt ttg gat gcc cgg gag gtg  
atc ccc atg gca gca gta aag caa gcg ctg agg gag gca ggc gac gag ttt gaa ctg cgg  
tac cgg cgg gca ttc agt gac ctg aca tcc cag ctc cac atc acc cca ggg aca gca tat  
cag agc ttt gaa cag gta gtg aat gaa ctc ttc cgg gat ggg gta aac tgg ggt cgc att  
gtg gcc ttt ttc tcc ttc ggc ggg gca ctg tgc gtg gaa agc gta gac aag gag atg cag  
gta ttg gtg agt cgg atc gca gct tgg atg gcc act tac ctg aat gac cac cta gag cct  
tgg atc cag gag aac ggc ggc tgg gat act ttt gtg gaa ctc tat ggg aac aat gca gca  
gcc gag agc cga aag ggc cag gaa cgc ttc aac cgc tgg ttc ctg acg ggc atg act gtg  
gcc ggc gtg gtt ctg ctg ggc tca ctc ttc agt cgg aaa tga