

ORIGINAL ARTICLE

Molecular targeting of Bcl-2 overcomes prostate cancer cell adaptation to XIAP gene downregulation

Y Nakano, V Bilim, K Yuuki, A Muto, T Kato, A Nagaoka and Y Tomita

Department of Urology, Yamagata University School of Medicine, Yamagata, Japan

X-linked inhibitor of apoptosis (XIAP) is a suppressor of apoptosis that supports an increased survival and resistance to chemotherapy of human prostate cancer (PCa) cells. Effects of transient (24 h) and chronic (beyond 1 month) downregulation of XIAP in DU145 hormone refractory prostate cancer (HRPC) cells were studied. We found that transient downregulation of XIAP by siRNAs resulted in an increase of apoptosis and a decrease in Bcl-2 levels and sensitized PCa cells to cisplatin. XIAP downregulation by shRNA vector stable transfection led to upregulation of Bcl-2 protein. Our results identify the adaptability of PCa cells to chronic loss of XIAP in part through upregulation of Bcl-2 and indicate that multitargeting approach is the most effective application in the chemotherapy of human HRPC.

Prostate Cancer and Prostatic Diseases (2009) 12, 34–40; doi:10.1038/pcan.2008.27; published online 13 May 2008

Keywords: XIAP; Bcl-2; siRNA; small-molecule inhibitor

Introduction

Prostate cancer (PCa) has become the sixth leading cancer in men in Japan (National Cancer Institute, Cancer Information Service) and the incidence is increasing steadily with an estimated 30 000 new cases yearly by 2015. PCa is the cause of 3.5% of cancer-related deaths in Japan, and the death rate due to PCa has increased ninefold during the past 30 years. In the United States, it is the second leading cause of cancer death in men. In cases of local recurrence or systemic disease, hormonal therapy is applied. However, it is palliative, ultimately resulting in the emergence of androgen-insensitive clones and death from the disease. Cytotoxic chemotherapy with taxanes has been demonstrating promising results but its effectiveness in patients with hormone refractory prostate cancer (HRPC) is far from satisfactory.^{1–3} New molecular targeting drugs are undergoing trials.^{4,5} So far, patients with HRPC have several treatment options but none of them has a great impact on survival. Thus, there is a vital necessity for new therapeutic modalities.

Emergence of HRPC is associated with continuous signaling through androgen receptors (ARs) even in the absence or decreased milieu of androgens. This is caused by altered expression of coregulators, mutation in ARs leading to relaxed affinity for various ligands or even

ligand-independent activation. HRPC has been also associated with abrogation of apoptosis and multiple changes in intracellular signaling pathways. DU145 cells do not harbor AR, distinguishing them from clinical HRPC. Nevertheless, these cells are independent of androgen and are one of the most popular *in vitro* models of HPRC.

Levels of inhibitor of apoptosis (IAP) family members were shown to be increased in PCa compared with normal prostate epithelium⁶ and were present in cultured PCa cell lines.⁷ Moreover, resistance to chemotherapeutic drugs and tumor-necrosis factor (TNF) family ligands was attributed to high levels of expression of IAP family members.^{8,9} The IAP family member survivin was also shown to interfere with hormonal therapy¹⁰ and X-linked inhibitor of apoptosis (XIAP) contributed to the resistance of circulating prostate cells to anoikis.¹¹ Targeting IAPs in PCa by antisense oligonucleotides resulted in a reversal of drug resistance.^{9,12} Acquired resistance is one of major problems in developing new treatment modalities. In the clinical setting, cancer treatment takes a long time and constitutive suppression of XIAP is a better *in vitro* model to characterize new treatment options. Constitutive depletion of antiapoptotic proteins in cancer cells can promote further changes leading to resistance rather than susceptibility to apoptosis. Thus, knockdown of Bcl-xL, another important antiapoptotic molecule in PCa cell lines, resulted in phenotypic changes and resistance to chemotherapeutic drugs.¹³

Recently, siRNA has emerged as a powerful means to downregulate gene expression. To the best of our knowledge, this is the first report on the use of siRNA to suppress XIAP in PCa cells. Furthermore, we

Correspondence: Professor Y Tomita, Department of Urology, Yamagata University School of Medicine, Iida-nishi 2-2-2, Yamagata 990-9585, Japan.

E-mail: ytomita@med.id.yamagata-u.ac.jp

Received 3 December 2007; revised 18 March 2008; accepted 11 April 2008; published online 13 May 2008

compared effects of acute (by siRNA) and constitutive (utilizing shRNA expression vector) depletion of XIAP in the DU145 HRPC cell line and studied limiting factors for possible future clinical applications of these modalities.

Materials and methods

Cell culture

An established HRPC cell lines, DU145 and PC3 as well as hormone-sensitive cell line LNCaP were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured as described previously.¹⁴ The cells were grown in RPMI 1640 medium (Gibco BRL, NY, USA) supplemented with 10% fetal calf serum (CSL Limited, Victoria, Australia), 1% MEM nonessential amino acids (Gibco BRL), 100 mM of 1% MEM sodium pyruvate solution (Gibco BRL) and 50 mg ml⁻¹ kanamycin (referred to as complete medium) in an incubator at 37 °C containing 5% CO₂. The small-molecule Bcl-2 inhibitor HA14-1, a ligand of a Bcl-2 surface pocket (molecular weight 409, micromolar affinity to Bcl-2¹⁵), was purchased from Calbiochem (San Diego, CA, USA), cisplatin was from Nippon Kayaku (Tokyo, Japan), and docetaxel was from Sigma-Aldrich Japan (Tokyo, Japan).

Immunoblotting

Immunoblotting was performed as described previously.¹⁶ horseradish peroxidase-labeled secondary antibody was detected using a SuperSignal West Pico Substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. β -Actin was used as a loading control. The images were analyzed using UN-SCAN-Itgel Automated Digitizing System software (version 5.1 for Windows, Silk Scientific Inc., Orem, UT, USA). The following antibodies were used: anti-XIAP (clone 2F1, MBL, Nagoya, Japan), anti-Bcl-2 (clone 124, Dako, Japan), anti-Bcl-X, anti-cIAP1, anti-cIAP2 and anti-p65 nuclear factor- κ B (NF- κ B) were from Becton Dickinson (Franklin Lakes, NJ, USA).

RNA extraction, real-time RT-PCR and microarray analysis

Total cellular RNA was extracted using the SV total RNA Isolation System (Promega, Madison, WI, USA) and the first-strand DNA was synthesized using a cDNA Reverse Transcription Kit (Applied Biosystems Japan, Tokyo, Japan) following the manufacturer's instructions. Real-time quantitative RT-PCR was performed in the 7300 Real Time PCR System (Applied Biosystems Japan). We used predesigned TaqMan Gene Expression Assays (Applied Biosystems Japan) targeting human *Bcl-2* (Hs00236808_s1) mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 4352934E) as an endogenous control. Each experiment was repeated three times to confirm reproducibility with the reaction in triplicate wells for each sample using a TaqMan Universal PCR Master Mix (Applied Biosystems Japan) according to the standard protocol. The expression of the target mRNA was quantified relative to that of the

GAPDH mRNA and parental cells or untreated controls were used as a reference.

Expression profiling was completed using Affymetrix GeneChip Human Genome U133 plus 2.0 Array microarrays. The data were subsequently analyzed by using the GeneSpring GX Workgroup and Viewer software package.

RNA interference

Three XIAP-targeting shRNA vectors were generated using pcPURU6 β -i-cassette with the target sequences 5'-GTAGAAGAGTTTAATAGAT-3' (TA0025-1), 5'-GCCG GAATCTTAATATTCG-3' (TA0025-2) and 5'-AGGTGA AGGTGATAAAGTA-3' (TA0025-4) by the TaKaRa Bio company (Shiga, Japan). Transfection was carried out using TransIT-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI, USA). In preliminary experiments, TA0025-4 demonstrated the highest ability to suppress XIAP and was selected for further experiments. For the generation of stable transfectant clones, the transfected cells were selected with puromycin for 3–4 weeks. Twenty selected clones were screened for XIAP expression and clones 3 and 10 with the lowest levels of XIAP (32 and 2.3% to control, respectively) were selected for further experiments. We also isolated puromycin-resistant mock transfectants, produced by transfection of a vector carrying an irrelevant sequence (5'-CACCTTT TTTT-3') with no mammalian homology. Transient treatment was carried out using two XIAP-specific siRNAs TOMY and KON produced by the TaKaRa Bio company (Shiga, Japan; the sequences were not disclosed by the supplier) with the help of TransIT-TKO Transfection Reagent (Mirus Bio Corporation). Final concentration of siRNAs in the medium was 10 nM.

Measurement of cell viability

Cell viability was detected with a colorimetric assay, the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) utilizing tetrazolium compound according to the manufacturer's instructions. Each dose was examined in four or six wells of 96-well plate (BD Falcon, Becton Dickinson) and the mean and standard deviation were calculated. Each experiment was repeated at least three times and representative results are presented.

Detection of apoptosis

Cells were cultured in Lab-Tek Chambers (Nunc Inc., Naperville, IL, USA), treated with siRNA and/or cisplatin. Apoptotic morphological changes were detected with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) staining followed by observation under phase contrast and fluorescence microscopes.

Statistical analysis

Continuous variables are presented as the mean \pm s.d. Continuous variables in the present study met the criteria for a normal distribution and were assumed to be parametric. They were analyzed using a two-tailed *t*-test or one-way analysis of variance (ANOVA, with a posttest to compare all pairs of values) where appro-

appropriate. *P*-values lower than 0.05 were considered statistically significant. Analysis was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

DU145 expressed the highest levels of XIAP among examined PCa cell lines

Among the three PCa cells examined LNCaP, hormone-sensitive cell line, expressed the lowest levels of XIAP, and DU145 showed the highest amount of XIAP (relative density (XIAP/actin) was 8 times higher than in LNCaP and 1.5 times higher than in PC3). Moreover, cIAP-2 protein was detected only in DU145 (Figure 1).

Transient knockdown of XIAP by siRNA sensitized DU145 cells to cisplatin

The transient transfection of DU145 cells with XIAP-targeting siRNAs for 24 h produced a prominent downregulation of XIAP expression (to 46.5% by KON and 22.7% by TOMY; Figure 2a). XIAP was knocked down to undetectable levels at 48 and 72 h after transfection (data not shown). Pretreatment with either KON or TOMY resulted in a slight but significant ($P < 0.01$) decrease in the number of cells (Figure 2b) accompanied by an appearance of cleaved poly-(ADP-ribose)polymerase (PARP; Figure 2c). Both siRNA sequences also sensitized the cells to cisplatin ($P < 0.0001$; Figure 3a), but did not exert any effect on susceptibility to docetaxel (Figure 3b). The decrease in cell numbers and appearance of morphologically typical apoptotic cells were confirmed by examining Hoechst 33342 micrographs (Figure 4).

Clones with stable downregulation of XIAP were more sensitive to cisplatin than parental and mock cells

To explore the effect of a constitutive decrease in the expression of XIAP on cell survival and susceptibility to apoptosis, we generated clones of DU145 PCa cells stably transfected with an shRNA expressing vector-targeting XIAP (TA0025-4) as well as a control vector. Twenty clones were selected for screening. XIAP protein was suppressed by 68% in clone 3 and by 97.7% in clone 10 (Figure 5a), and these two clones were selected for further experiments. Clones 3 and 10, as well as the mock clone, did not demonstrate any morphological difference from the parental cells (data not shown). As XIAP was

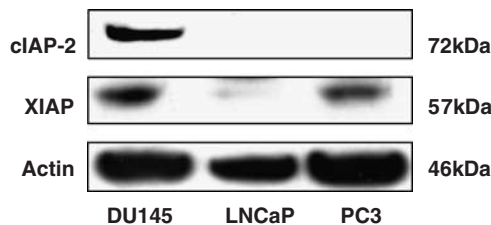


Figure 1 Western blot analysis of X-linked inhibitor of apoptosis (XIAP) and cIAP-2 using three prostate cancer (Pca) cell lines, DU145, LNCaP and PC3.

linked not only to apoptosis but also to cell proliferation,¹⁷ we analyzed the doubling time of the parental, mock and transfectant clones. Proliferation rates did not differ between the two transfectants, mock transfectant and parental cells (data not shown). As in the case of transient transfection, the cells demonstrated equal sensitivity to docetaxel (data not shown), but unexpectedly, resistance to cisplatin was increased in clone 10 followed by clone 3, with equal sensitivity in the parental and mock clones (Figure 5b).

Bcl-2 expression increased in cells with stable downregulation of XIAP

To search for the cause of the increased resistance to cisplatin we examined a panel of apoptosis-related genes by performing a western blot analysis for a panel of Bcl-2 and IAP family members. The only protein showing changes in clones 3 and 10 was Bcl-2 (Figure 6a), increasing 4.3- and 8.29-fold, respectively. *Bcl-2* mRNA also increased significantly in these clones (Figure 6b).

To search for a possible explanation of this phenomenon, we examined *Bcl-2* expression in transient transfectants. *Bcl-2* protein was reduced to 50.4 and 35.0% (Figure 7a) and the mRNA was decreased to 49.1 and 59.5%, respectively at 24 h (Figure 7b). Longer transfection up to 72 h did not demonstrate any increased of

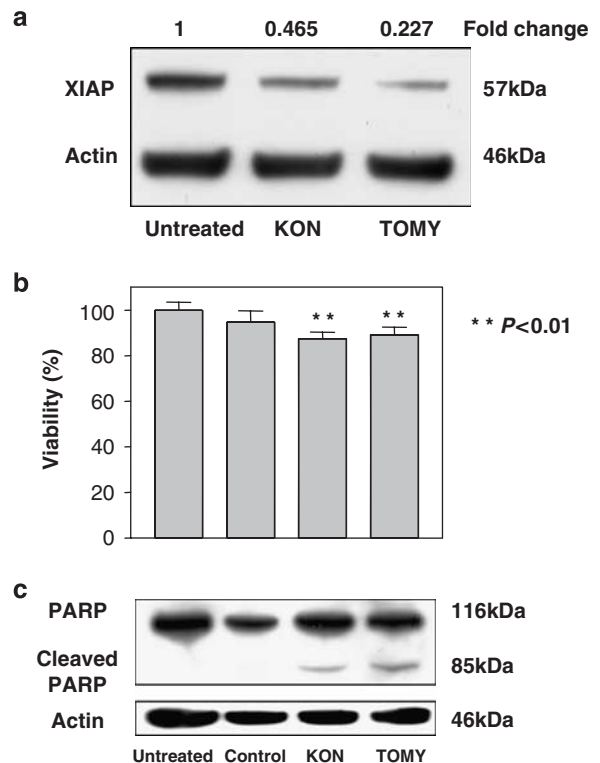


Figure 2 Western blot analysis of X-linked inhibitor of apoptosis (XIAP) (a, figures above indicate fold change of protein levels) and poly-(ADP-ribose)polymerase (PARP) (c); cleaved PARP product was detected after XIAP knockdown with siRNA (KON and TOMY sequences). MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) viability assay (b) of untreated cells and cells transiently transfected for 24 h with either of the two XIAP-targeting siRNA sequences (KON, TOMY, final concentration 10 nM) DU145 cells.

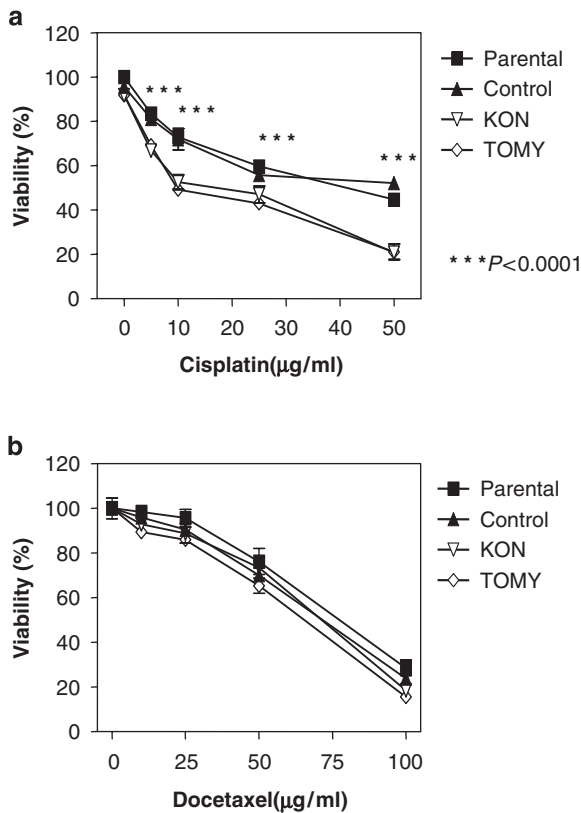


Figure 3 MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) viability assay of DU145 cells transiently transfected with X-linked inhibitor of apoptosis (XIAP) siRNAs (final concentration 10 nM) for 24 h and posttreated with indicated concentrations of cisplatin (a) or docetaxel (b) for another 24 h. For statistical analysis one-way analysis of variance (ANOVA) was applied with a Tukey's multiple comparison posttest (difference was found between parental/control and KON/TOMY); *** $P < 0.001$.

Bcl-2 either (data not shown). Other IAP family members like c-IAP1 and c-IAP2 as well as Bcl-x's splice variants were not changed (data not shown). The NF- κ B p65 subunit also decreased to 20.2 and 13.0% compared to the control baseline after treatment with KON and TOMY, respectively (Figure 7c), although it was unchanged in stable clones (Figure 6a).

To estimate the functional significance of Bcl-2 overexpression in stable clones, cells were treated with HA14-1, a small-molecule Bcl-2 inhibitor. Apoptosis was induced more prominently in clone 10 with the highest levels of Bcl-2 among the cells examined (Figure 6c).

Microarray analysis

To expand the search for putative genes, a microarray analysis was performed. Changes of gene expression observed in mock cells compared with parental cells were elucidated to be nonspecific and those genes were excluded from further analysis. The expression of 360 and 287 genes increased twofold or more in clone 3 and clone 10, respectively. Among them, expression of 37 genes increased in both clones 3 and 10. The number of apoptosis-related genes was 24, 12 and 2, respectively (Table 1). The expression of 995 genes in clone 3 and 826 genes in clone 10 decreased by half or more compared

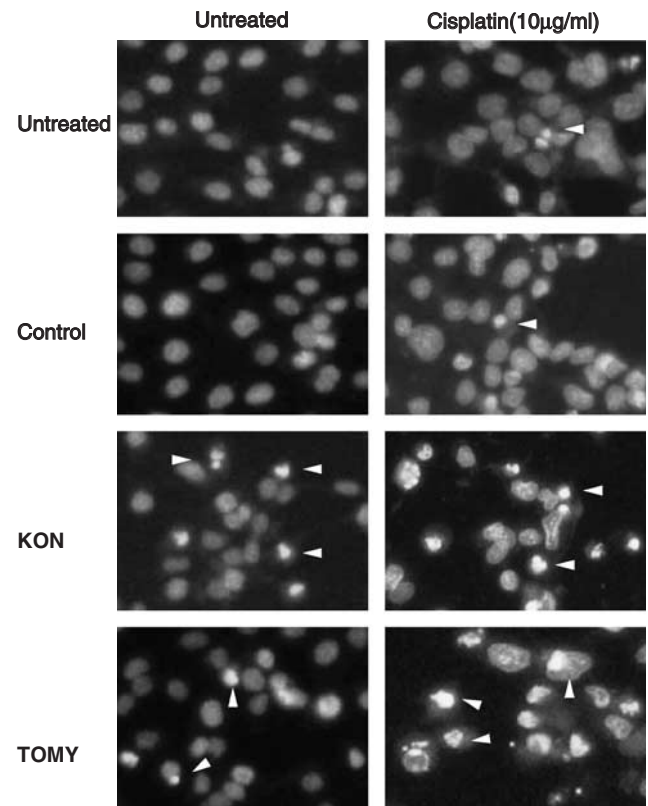


Figure 4 Hoechst 33342 photo of DU145 cells treated for 24 h with X-linked inhibitor of apoptosis (XIAP) siRNA only (final concentration 10 nM, left) or posttreated with $10 \mu\text{g ml}^{-1}$ cisplatin for 24 h (right). DU145 cells were either left untreated, or treated with transfection reagent only or either of the two siRNA sequences. The photos were taken using Axiovert 200 Zeiss microscope at $\times 100$ magnification. Typical apoptotic cells containing condensed and fragmented nuclei are pointed with arrowheads.

with parental cells. Among them, expression of 189 genes decreased in both clones 3 and 10. The number of apoptosis-related genes was 30, 29 and 7, respectively (Table 2).

It is obvious that the total number of genes whose expression decreased exceeded the number of genes whose expression increased; however, this difference was not prominent among apoptosis-related genes. Genes downregulated in both clones after XIAP shRNA stable transfection were mainly related to TNF α family-related signaling, including Fas, caspase-8 apoptosis regulator, TNF α -induced protein and phosphoinositide-3 kinase regulatory subunit (Table 2). The two genes that were upregulated twofold or more in both clones (Table 1) were Fas-activated serine/threonine kinase and Cullin 4A, last is known to be involved in ubiquitination and subsequent proteasomal degradation of target proteins, particularly in response to radiation-induced DNA damage. It is interesting that clusterin, a protein notorious for resistance to apoptosis in HRPC, was upregulated in clone 10.

Discussion

Docetaxel-based regimens are the new standard for treating advanced HRPC.¹⁸ Although platinum-based

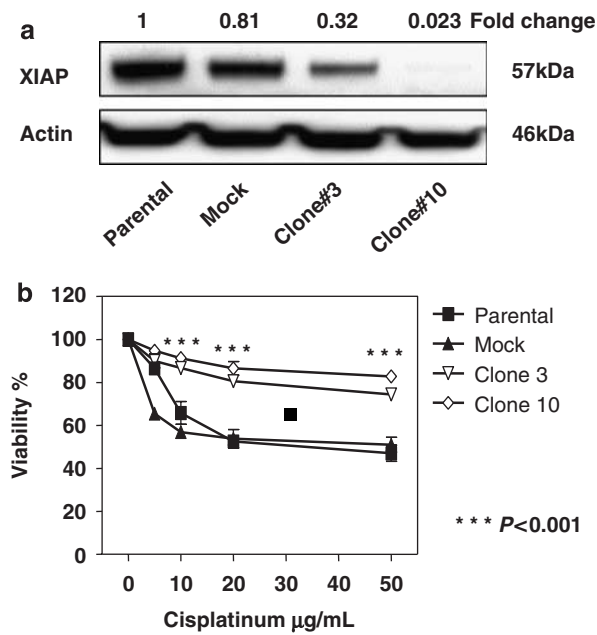


Figure 5 Immunoblotting for X-linked inhibitor of apoptosis (XIAP) (a, figures above indicate fold change of protein levels) after stable transfection of XIAP shRNA vector into DU145 cells. β -Actin was applied as a control for loading. Figures above the blots represent relative values of band densitometry. MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) cell viability assay of XIAP-targeting stable transfectant clones as well as parental cells and mock transfectants treated with indicated concentrations of cisplatin (b) for 24 h.

chemotherapy drugs have a moderate level of clinical activity against HRPC, recent clinical trials revealed that they may have potential as second-line chemotherapy drugs.¹⁹

Earlier, we demonstrated the importance of XIAP to the malignant potential of transitional cell cancer (TCC) and chemosensitization of TCC by XIAP antisense oligonucleotide.²⁰ A decrease in levels of XIAP has been also identified to be a key factor in cisplatin sensitivity and XIAP antisense oligonucleotide-potentiated cisplatin sensitivity in DU145 PCA cells.⁹ siRNA technology is much more able to suppress target genes than antisense oligonucleotides and, to our knowledge, has not been previously applied to XIAP in PCA cells. The transient transfection of DU145 cells with two different XIAP-targeting siRNAs produced a prominent downregulation of XIAP (Figure 2a) that *per se* resulted in apoptosis (Figures 2b, c, 4a and b) and significantly increased sensitivity to cisplatin ($P < 0.0001$; Figures 3a, 4a and b).

One of the features of cancer cells is their adaptability, which can interfere with treatment modalities. Clinical response can be achieved only by continuous suppression of cell proliferation and constant inhibition of prosurvival molecules in cancer cells. It has been reported that constitutive antisense RNA downregulation of Bcl-xL in PCA cells led to resistance to cytotoxic agents, a decrease in the growth rate and a drastic upregulation of XIAP expression.¹³ Here we generated and characterized XIAP shRNA stable clones of DU145. In viability assay clones 3 and 10 unexpectedly demonstrated increased resistance to cisplatin with a significant

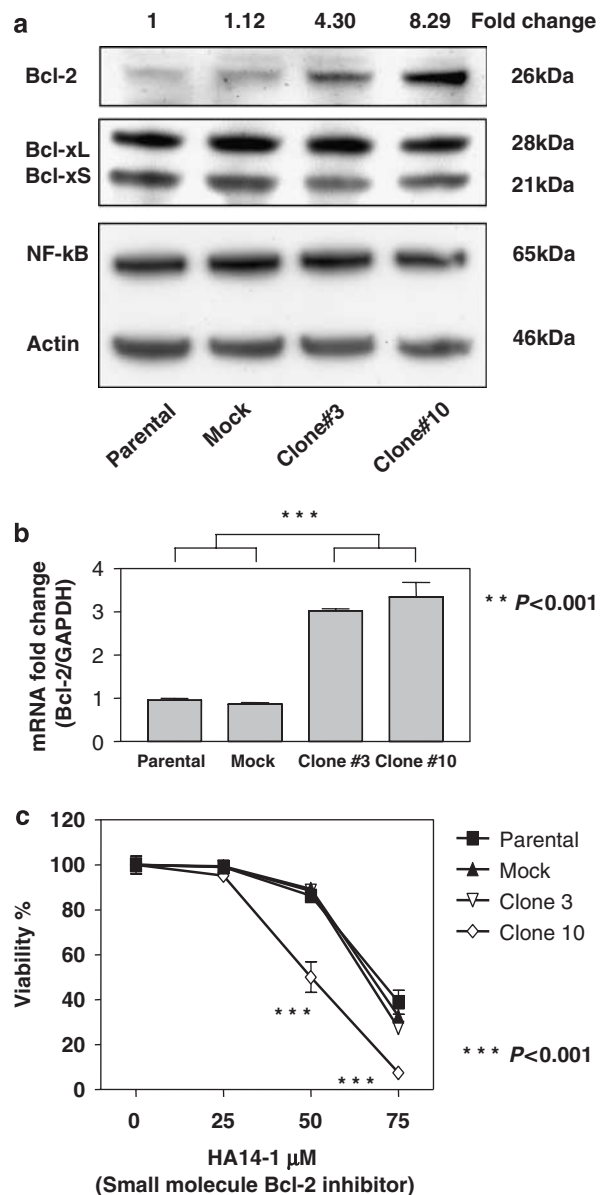


Figure 6 Immunoblotting for Bcl-2 (figures above indicate fold change of protein levels), Bcl-x and nuclear factor- κ B (NF- κ B) (a) in stably transfected clones. β -Actin was applied as a control for loading. Figures above the blots represent relative values of Bcl-2 band densitometry. Fold change of relative Bcl-2 mRNA expression (calculated as a ratio of Bcl-2/glyceraldehyde-3-phosphate dehydrogenase, GAPDH) (b) detected by real-time RT-PCR utilizing TaqMan technology. MTS cell viability assay of X-linked inhibitor of apoptosis (XIAP)-targeting stable transfectant clones as well as parental cells and mock transfectants treated with indicated concentrations of HA14-1 Bcl-2 small-molecule inhibitor (c) for 24 h.

difference in the IC₅₀ among clones 3, 10 and the mock or parental clones (Figure 5b).

We analyzed the expression of Bcl-2 and XIAP families of antiapoptotic proteins searching for an explanation of this phenomenon. Bcl-2 increased at both the mRNA and protein levels (Figure 6a) whereas there was no change in the other molecules examined (Figure 6a; data not shown). Then we examined the status of Bcl-2 and its family members in transiently transfected cells. Bcl-2 protein and mRNA levels decreased in the transiently

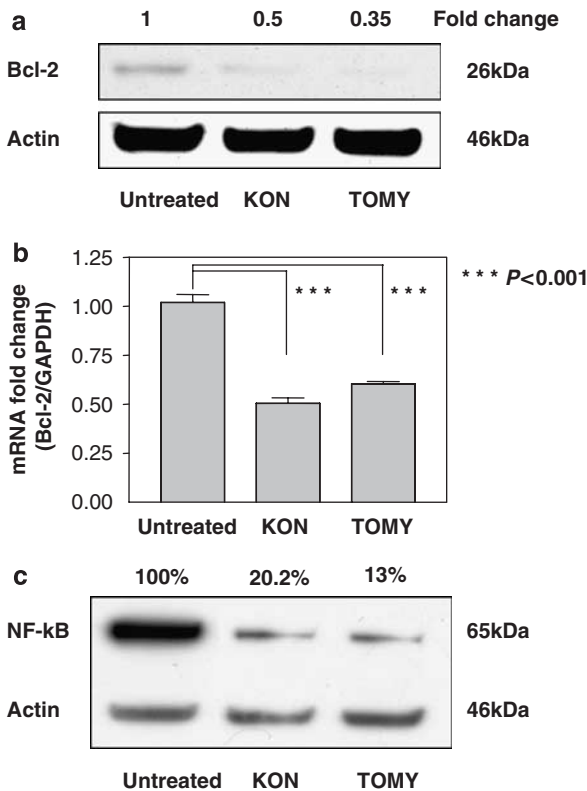


Figure 7 Immunoblotting for Bcl-2 (a, figures above indicate fold change of protein levels) and nuclear factor- κ B (NF- κ B) (c) after transient transfection of siRNA into DU145 cells. β -Actin was applied as a control for loading. Figures above the blots represent relative values of band densitometry. Fold change of relative Bcl-2 mRNA expression (calculated as a ratio of Bcl-2/glyceraldehyde-3-phosphate dehydrogenase, GAPDH) (b) detected by real-time RT-PCR utilizing TaqMan technology.

Table 1 Apoptosis-related genes, which expression increased two times and more in both clones 3 and 10

Gene name	Description
227757_at	Cullin 4A
210975_x_at	Fas-activated serine/threonine kinase

transfected cells (Figure 7a). Both Bcl-x splice variants, as well as c-IAP1 and c-IAP2 proteins, were unchanged. The decrease of Bcl-2 can be explained as either a nonspecific or specific indirect effect of XIAP siRNAs. The last hypothesis is supported by the finding that the two different siRNA sequences (KON and TOMY) had a similar effect on Bcl-2 and Bcl-x expression and the last was not modulated by this treatment. The downregulation of Bcl-2 was altered at the transcriptional level, and not by accelerated degradation, as confirmed by quantitative RT-PCR (Figure 7b). We tried to look for potential mechanisms of this phenomenon. It has been reported on the presence of the autoregulation of XIAP NF- κ B loop.^{17,21,22} Bcl-2 is one of the downstream genes of NF- κ B.²³ Thus, XIAP downregulation may result indirectly in decreased Bcl-2 expression. In fact, XIAP siRNA treatment produced a reduction in NF- κ B protein (Figure 7c).

Table 2 Apoptosis-related genes, which expression decreased to one-half or lower in both clones 3 and 10

Gene name	Description
219209_at	Interferon induced with helicase C domain 1
202643_s_at	Tumor-necrosis factor (TNF)- α -induced protein 3
212240_s_at	Phosphoinositide-3-kinase, regulatory subunit 1 (p85- α)
211317_s_at	CASP8 and FADD-like apoptosis regulator
211527_x_at	Vascular endothelial growth factor A
216252_x_at	Fas (TNF receptor superfamily, member 6)
209310_s_at	Caspase 4, apoptosis-related cysteine peptidase

Different from transient treatment with XIAP siRNAs, Bcl-2 was upregulated even in the unchanged milieu of the p65 NF- κ B subunit (Figure 6a). To check the functional effect of Bcl-2 inhibition on the stable transfectants, we treated the cells with HA14-1, a small-molecule Bcl-2 inhibitor, which induced apoptosis more prominently in clone 10 with the highest levels of Bcl-2 and the maximum suppression of XIAP among the cells examined. The reason that clone 3 was less resistant to HA14-1 may be the amount of Bcl-2 protein (roughly one half of that in clone 10) and suppressed but still detectable levels of XIAP.

Looking for other candidates responsible for the increased resistance of stable clones, we performed a microarray analysis. Some proapoptotic genes were downregulated and others upregulated in clones 3 and 10. However, it is difficult to draw a conclusion as to the cause of the resistance of these clones to apoptosis based on the results of a microarray analysis. Some molecules that demonstrated drastic changes in their protein levels, like Bcl-2, did not surpass the twofold cutoff in the microarray analysis, although real-time RT-PCR showed threefold rise in Bcl-2 mRNA. Many proteins are known to undergo posttranscriptional regulation, like IAP family members, whereas others experience posttranslational modifications that result in stability or gain/loss of function.

In contrast to cisplatin, docetaxel induced cell death to the same degree in the parental cells, transient transfectants as well as mock transfectants, and clones 3 and 10 (Figure 3b; data not shown). There is strong evidence of docetaxel efficacy in HRPC.²⁴ Docetaxel is a taxoid that inhibits the depolymerization of microtubules, which disturbs the normal mitotic machinery and leads to aberrant mitosis and cell-cycle arrest²⁵ with apoptosis. However, docetaxel has another mechanism of inducing apoptosis by serine phosphorylating Bcl-2,²⁵⁻²⁸ which ultimately results in its inhibition and apoptosis. The fact that parental cells and transfectants differ in sensitivity to cisplatin and docetaxel is of great interest. The examination of Bcl-2 phosphorylation status following the treatment with the two compounds exceeds the scope of the present paper and an additional investigation is deemed to be necessary for a thorough study of this phenomenon.

Conclusion

XIAP downregulation is a potent means to overcome the resistance of HRPC to chemotherapeutic agents. XIAP

downregulation also results in a decrease of Bcl-2 presumably via NF- κ B suppression. However, long-term XIAP downregulation produces an upregulation of Bcl-2 by a poorly understood mechanism and rebound in drug resistance. Further study of the link between IAP and Bcl-2 is necessary, but the possibility of adaptation to long-term exposure for XIAP siRNA should be kept in mind while developing new therapeutic agents for HRPC.

Acknowledgements

This research was supported in part by Grants-in-Aid for Scientific Research (no. 17390435) from the Ministry of Education, Science and Culture, Japan (to Y Tomita).

References

- Lucas A, Petrylak DP. The case for early chemotherapy for the treatment of metastatic disease. *J Urol* 2006; **176**: S72–S75.
- Gilbert DC, Parker C. Docetaxel for the treatment of prostate cancer. *Future Oncol* 2005; **1**: 307–314.
- Fossa SD, Jacobsen AB, Ginman C, Jacobsen IN, Overn S, Iversen JR et al. Weekly docetaxel and prednisolone versus prednisolone alone in androgen-independent prostate cancer: a randomized phase II study. *Eur Urol* 2007; **52**: 1691–1699.
- Carducci MA, Jimeno A. Targeting bone metastasis in prostate cancer with endothelin receptor antagonists. *Clin Cancer Res* 2006; **12**: 6296s–6300s.
- Uemura H, Nakaigawa N, Ishiguro H, Kubota Y. Antiproliferative efficacy of angiotensin II receptor blockers in prostate cancer. *Curr Cancer Drug Targets* 2005; **5**: 307–323.
- Krajewska M, Krajewski S, Banares S, Huang X, Turner B, Bubendorf L et al. Elevated expression of inhibitor of apoptosis proteins in prostate cancer. *Clin Cancer Res* 2003; **9**: 4914–4925.
- McEleny KR, Watson RW, Coffey RN, O'Neill AJ, Fitzpatrick JM. Inhibitors of apoptosis proteins in prostate cancer cell lines. *Prostate* 2002; **51**: 133–140.
- Ng CP, Bonavida B. X-linked inhibitor of apoptosis (XIAP) blocks Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis of prostate cancer cells in the presence of mitochondrial activation: sensitization by overexpression of second mitochondria-derived activator of caspase/direct IAP-binding protein with low pl (Smac/DIABLO). *Mol Cancer Ther* 2002; **1**: 1051–1058.
- Amantana A, London CA, Iversen PL, Devi GR. X-linked inhibitor of apoptosis protein inhibition induces apoptosis and enhances chemotherapy sensitivity in human prostate cancer cells. *Mol Cancer Ther* 2004; **3**: 699–707.
- Zhang M, Latham DE, Delaney MA, Chakravarti A. Survivin mediates resistance to antiandrogen therapy in prostate cancer. *Oncogene* 2005; **24**: 2474–2482.
- Berezovskaya O, Schimmer AD, Glinskii AB, Pinilla C, Hoffman RM, Reed JC et al. Increased expression of apoptosis inhibitor protein XIAP contributes to anoikis resistance of circulating human prostate cancer metastasis precursor cells. *Cancer Res* 2005; **65**: 2378–2386.
- McEleny K, Coffey R, Morrissey C, Williamson K, Zangemeister-Wittke U, Fitzpatrick JM et al. An antisense oligonucleotide to cIAP-1 sensitizes prostate cancer cells to fas and TNF α mediated apoptosis. *Prostate* 2004; **59**: 419–425.
- Vilenchik M, Raffo AJ, Benimetskaya L, Shames D, Stein CA. Antisense RNA down-regulation of bcl-xL expression in prostate cancer cells leads to diminished rates of cellular proliferation and resistance to cytotoxic chemotherapeutic agents. *Cancer Res* 2002; **62**: 2175–2183.
- Tomita Y, Bilim V, Hara N, Kasahara T, Takahashi K. Role of IRF-1 and caspase-7 in IFN- γ enhancement of Fas-mediated apoptosis in ACHN renal cell carcinoma cells. *Int J Cancer* 2003; **104**: 400–408.
- Wang JL, Liu D, Zhang ZJ, Shan S, Han X, Srinivasula SM et al. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci USA* 2000; **97**: 7124–7129.
- Tomita Y, Kawasaki T, Bilim V, Takeda M, Takahashi K. Tetrapeptide DEVD-aldehyde or YVAD-chloromethylketone inhibits Fas/Apo-1(CD95)-mediated apoptosis in renal-cell-cancer cells. *Int J Cancer* 1996; **68**: 132–135.
- Levkau B, Garton KJ, Ferri N, Kloke K, Nofer JR, Baba HA et al. XIAP induces cell-cycle arrest and activates nuclear factor- κ B: new survival pathways disabled by caspase-mediated cleavage during apoptosis of human endothelial cells. *Circ Res* 2001; **88**: 282–290.
- Eymard JC, Priou F, Zannetti A, Ravaud A, Lepille D, Kerbrat P et al. Randomized phase II study of docetaxel plus estramustine and single-agent docetaxel in patients with metastatic hormone-refractory prostate cancer. *Ann Oncol* 2007; **18**: 1064–1070.
- Oh WK, Tay MH, Huang J. Is there a role for platinum chemotherapy in the treatment of patients with hormone-refractory prostate cancer? *Cancer* 2007; **109**: 477–486.
- Bilim V, Kasahara T, Hara N, Takahashi K, Tomita Y. Role of XIAP in the malignant phenotype of transitional cell cancer (TCC) and therapeutic activity of XIAP antisense oligonucleotides against multidrug-resistant TCC *in vitro*. *Int J Cancer* 2003; **103**: 29–37.
- Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J, de Martin R. Activation of NF- κ B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. *J Biol Chem* 2000; **275**: 22064–22068.
- Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)- κ B-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med* 1998; **188**: 211–216.
- Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. *Oncogene* 2001; **20**: 7342–7351.
- Petrylak DP. Future directions in the treatment of androgen-independent prostate cancer. *Urology* 2005; **65**: 8–12.
- Moos PJ, Fitzpatrick FA. Taxanes propagate apoptosis via two cell populations with distinctive cytological and molecular traits. *Cell Growth Differ* 1998; **9**: 687–697.
- Haldar S, Chintapalli J, Croce CM. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res* 1996; **56**: 1253–1255.
- Haldar S, Jena N, Croce CM. Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci USA* 1995; **92**: 4507–4511.
- Fang G, Chang BS, Kim CN, Perkins C, Thompson CB, Bhalla KN. 'Loop' domain is necessary for taxol-induced mobility shift and phosphorylation of Bcl-2 as well as for inhibiting taxol-induced cytosolic accumulation of cytochrome c and apoptosis. *Cancer Res* 1998; **58**: 3202–3208.