Chapter 1

Mechanisms by Which Non-Targeted Genes Compensate for Specific Gene Therapy Directed Towards Bcl-2 in a Prostate Cancer Model

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Abstract

Antisense oligonucleotides (oligos) have targeted regulatory proteins in both *in vivo* and *in vitro* prostate cancer models featuring both the LNCaP and PC-3 cell lines. In efforts to identify compensatory changes in the expression of non-targeted genes these studies evaluated mono- and bispecific oligos capable of targeting and equally suppressing the expression of bcl-2 (an apoptosis inhibitor). Effects upon non-targeted genes could potentially lead to development of more phenotypically aggressive and metastatic tumors. The summation presented here demonstrate that oligo treated LNCaP cells compensate for diminished bcl-2 by suppressing caspase-3 (an apoptosis promoter) while enhancing the expression of AKT-1 (another apoptosis inhibitor). In addition, we found enhanced expression of the androgen receptor (AR), its p300 and IL-6 co-activators, polymerase transcription mediator MED-12, and growth

regulating signal transducer STAT-3. Therefore, therapeutic approaches to restore apoptosis through suppression of bcl-2 lead to altered protein expression of non-targeted genes not only involving apoptosis, but also androgen sensitivity and transcription, and more. In addition, proteins associated with cell division as indicated by increased expression of the KI-67 proliferation antigen and mitosis regulating cyclin D1 are increased as are TMPRSS22 and FLI-1, associated respectively with gene fusion involving the ERG protein, as well as chromosomal instability. The net result is an altered pattern of gene expression often associated with more aggressive and proliferative tumors.

Keywords

Antisense; bcl-2; Prostate Cancer; Gene Therapy; Compensation

Introduction

In theory, gene therapy is specific but, in reality, it encounters difficulties in practice. While suitable targets are found in many pathways, and tumors express altered patterns of gene activity, the actual transcription activity of most genes which regulate tumor growth is comparable to those of normal cells. Resistance develops because the biochemical pathways involved are complex, frequently redundant and regulated to varying degrees by combinations of both stimulatory and inhibitory factors (as in apoptosis). Several years ago we noted that non-targeted genes are directly affected by what was believed to be spe-

cific therapy, and that some are susceptible to altered expression to an extent having the potential to reverse the effects of the originally intended treatment. With some genes, the altered pattern of gene expression can mimic those seen with more aggressive tumor types. This process of compensation [1] may drive tumors to become more aggressive, which in this (LNCaP) model involving cells of prostatic origin, appear to become more sensitive (than usual) to androgen and therefore potentially more aggressive. They also express substantially greater activity in proliferation antigen KI-67, as well as in the cyclin D1, a regulator of mitosis, suggesting an increase in tumor growth rates could occur following suppression of bcl-2, and the initial intent of restoring tumor cell apoptosis. Just as bacteria and viruses mutate to evade antibiotic and antiviral agents, tumor cells are under similar selective pressure to evade therapy, whether chemically induced (chemotherapy) or that based on suppression of gene translation (including antisense oligonucleotide [oligo] mediated gene therapy). While oligo based therapy is already entering the clinical environment, the unintended compensatory consequences of intervention are poorly understood, and could contribute to the emergence and selection of more aggressive cells through several mechanisms.

Gene therapy has been clinically employed for the treatment of human prostate tumors and together with radio- [2,3] or chemotherapy [4] antisense oligos have been administered against inhibitors of apoptosis (particularly

bcl-2 and clusterin) in attempts to increase this (often tumor suppressed and desirable) activity. In addition to oligo mediated suppression of bcl-2, Genentech has developed another type of inhibitor involving a bioavailable small molecule (venetoclax [GDC-0199/ABT-199]) which is currently in Phase I and II clinical trials for the treatment of a variety of tumor types.

In a number of previous studies (summarized in Table 1) we found that LNCaP cells treated with antisense oligos directed against bcl-2 (administered in a nanoparticle suspension of lipofectin) compensated by suppressing caspase-3 [1] (an apoptosis promoter), and enhancing AKT-1(an apoptosis inhibitor) [5], androgen receptor [6] (AR), and AR co-activators p300 [7] and IL-6 expression [8]. In addition, PD-1, its ligand PD-L1 (immune checkpoint blockage markers) and fas-ligand, which activate apoptosis through signal transduction, were also enhanced [9], as were suppressor protein p53 [10], oncogene v-myc [11], polymerase transcription mediator MED-12 [12] and signal transducer STAT-3 [5,12]. This suggests that (at least in this LNCaP model), therapeutic approaches to restore apoptosis (including the use of antisense oligos to suppress bcl-2) can lead to altered expression of non-targeted genes and regulatory changes not only involving apoptosis, but also androgen sensitivity, (suppressor/oncogene) transcriptional activity and immune responsiveness. Many of the androgen related alterations are similar

to the expression patterns associated with more advanced prostate tumors, while regulation of apoptosis involved both mitochondrial and signal transducing pathways. As noted, we demonstrated that cell surface proteins which regulate immune checkpoint blockade (PD-1 and PD-L1 and fas-ligand) and activate apoptosis via signal transduction were also enhanced [9], presumable leading to further inhibition of T-cell activity. Compensatory effects identified with these proteins are important since the PD-1/PD-L1 pathway is now recognized as a target for monoclonal antibody directed immunotherapy used to treat various solid tumors, particularly melanoma and lung tumors.

Numerous studies (Table 1) have evaluated compensatory effects upon apoptosis, androgen regulation, angiogenesis, autocrine, (oncogene/suppressor) transcriptional proteins, immunologic recognition, proliferation, mitosis, antigen expression, gene fusion products and chromosomal instability. We employed RT-PCR in these experiments to determine alterations in gene expression. Although more sophisticated techniques are available we find this method both sensitive enough to identify those genes involved with compensation, and able to identify non-targeted genes (like KI-67) which are particularly (and so far, most greatly) affected and could provide an additional target for oligos when used in combination with bcl-2 suppressive therapy.

Table 1: A summary of previous studies with relative changes and statistical significance noted

Protein	Treated MR2	Treated MR24	Treated MR42	Role	Special Category
	Monospecific	Bispecific	Bispecific		Conformation Dependent
Apoptosis					
Caspase-3	-35.80%	-40.30%	-43.50%	Promoter of apoptosis	
	0. 000203	0.000628	0.006013		
AKT-1	256.70%	189.40%	182.60%	Oncogene/ Inhibitor	
	0.000616	0.000425	0.002014	of apoptosis	
fas-ligand	88.60%	66.70%	75.80%	Initiator of apoptosis	
	0.00033	0.015039	0.001784		
PD-1	149.30%	320.70%	193.90%	Programmed death initiator	
	0.018476	0.034078	0.005988	Immune check-point inhibitor	
PD-L1	33.30%	51.00%	28.50%	Programmed death ligand	
	0.006079	0.014394	0.010793	Immune check-point inhibitor	
Androgen Sensitivity					
Androgen Receptor	31.20%	58.50%	53.10%	Androgen binding receptor	
	0.14869	0.019349	0.018608		
p300	82.90%	93.00%	105.40%	Androgen receptor co-activator	
	0.006297	0.044429	0.007257		
IL-6	236.90%	219.30%	139.20%	Androgen receptor co-activator/	
	0.001585	0.005231	0.001537	Cytokine	
Differentiation Antigen					
PSA	No change	No change	No change	Prostate Specific Antigen	No identified base pair complementarity in oligos
PSMA	No change	74.50%	71.10%	Prostate Specific	Bispecific oligos have
		0.03	0.02	Membrane Antigen	intrastrand base complementarity
PAP	No change	No change	No change	Prostatic Acid Phosphatase	No identified base pair complementarity in oligos
PCA-3	No change	No change	No change	Prostate Cancer Antigen-3	No identified base pair
				RNA molecule secreted in urine	complementarity in oligos
CD44	No change	No change	No change	Stem cell marker	No identified base pair complementarity in oligos
Interferon-gamma	No change	61.30%	24.50%	Inducible cytokine involved with	Bispecific oligos have
		0.005831	0.002701	cell surface antigen expression	intrastrand base complementarity
Growth/ Proliferation marker					
KI-67	363.00%	427.15	355.70%	Proliferation marker	
	0.000229	0.001137	0.002017		
Cyclin D1	51.20%	73.30%	33.7	Regulates cell cycle	
	0.021419	0.032361	0.048313		
STAT-3	166.20%	110.90%	37.80%	Signal transducer regulating growth	
	0.009439	0.004279	0.015529		
MED-12	138.10%	181.30%	No change	Polymerase transcription mediator	
	0.018239	0.022919	NS	of growth	
Onco/Suppressor gene					
v-myc	170.10%	201.40%	No change	Oncogene	
	0.002741343	0.000641946	NS		
p53	47.50%	86.50%	58.00%	Suppressor gene	
	0.021376	0.00013	0.007671		
Chromosomal Instability/gene fusion proteins					
TMPRS22	29.8%	19.0%	45.4%	Fusion protein with ERG	
	0.002939	0.022816	0.039764		
FLI-l	50.2%	48.5%	82.0%	Gene fusion transcription factor	
	0.006923	0.009585	0.00024		

Chromosomal instability and the production of fusion proteins are often associated with proliferating cancers. That's why increased activity of KI-67, TMPSS22 and FLI-1 are probably the best indicators of how gene therapy can be compromised or overcome. Fusion proteins from TMPSS22 and ERG are found in 40-80% of human prostate tumors, and ERG over expression is thought to disrupt AR signaling contributing to the development of androgen independent tumors. FLI-1 is a transcription factor associated with ETS expression and with the gene fusions increasingly found to be associated with development and progression of prostate cancer.

Methods

Oligonucleotides

Oligos (mono- or bispecific) were purchased from Eurofins MWG Operon (Huntsville, AL). Each was phosphorothioated on three terminal bases at 5' and 3' positions. Stock solutions were made to a final concentration of 625 μM in sterile Dulbecco PBS.

Base Sequences

Each oligo contained at least one **CAT** sequence and targeted the area adjacent to the mRNA AUG initiation codon for the respective targeted protein (EGFR or bcl-2).

MR$_4$ (monospecific targeting bcl-2) T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-**C-A-T**

MR_{24} (bispecific targeting EGFR/bcl-2) G-A-G-G-G-T-C-G-C-A-T-C-G-C-T-G-C-T-C-T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T

MR_{42} (bispecific targeting bcl-2/EGFR) T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-**C-A-T**-G-A-G-G-G-T-C-G-**C-A-T**-C-G-C-T-G-C-T-C

Cell Culture

LNCaP cells were grown in RPMI 1640 supplemented with 10% bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a 5% CO_2 incubator. Log phase cells were harvested using EDTA/trypsin and equally distributed into 75 cm^2 flasks (Corning, NY). At intervals media were either supplemented or replaced with fresh.

Oligo Treatment Prior to PCR

Four days prior to oligo addition, when cell density approached 75% confluence, 10 ml of fresh media was added. Cells were incubated for an additional 3 days before 5 ml of media was replaced with fresh the day before oligos were added. 100 µl of stock oligos were added to bring the final concentration to 6.25 µM. Incubation proceeded for an additional 24 hours in the presence or absence of monospecific MR_4, or the MR_{24} and MR_{42} bispecifics in a nanoparticle suspension with lipofectin.

RNA Extraction

Following treatment, media was removed, a single ml of cold (4°C) RNAzol B was added to each 75 cm^2 culture flask and the monolayer lysed by repeated passage through a pipette. All procedures were performed at 4°C. The lysate was removed, placed in a centrifuge tube to which 0.2 ml of chloroform was added, and shaken. The mixture stayed on ice for 5 min, was spun at 12,000 g for 15 min, and the upper aqueous volume removed and placed in a fresh tube. An equal volume of isopropanol was added, the tube shaken, and allowed to stay at 4°C for 15 min before similar centrifugation to pellet the RNA. The supernatant was removed, the pellet washed in a single ml of 75% ethanol, then spun for 8 min at 7500 g. The ethanol was pipetted off and the formed pellet air dried at -20°C.

RNA Quantitation

RNA was resuspended in 250 µl of DEPC treated H_2O, and quantitated using a Qubit fluorimeter and Quant-iT RNA assay kit (Invitrogen). DEPC is an inhibitor of RNase activity.

RT-PCR

Extracted RNA was diluted in DEPC treated water to 40 µg/µl. 1-4 µl of this RNA was added to 1 µl of both sense and antisense primers (forward and reverse sequences) for actin, bcl-2 and KI-67. The same procedure was followed to obtain the data listed in Table 1, which summarizes

our finding obtained from evaluating many non-targeted genes. Only those which demonstrated compensation are listed; those which were either poorly expressed or not affected are not. From a kit purchased from Invitrogen the following reactants were added for RT-PCR: 25 µl of 2X reaction mixture, 2 µl SuperScript III RT / platinum *Taq* mix, tracking dye, and 3 µl $MgSO_4$ (of a 5mM stock concentration). DEPC treated water was added to yield a final volume of 50 µl. RT-PCR was performed for 2 X 25 cycles using the F54 program in a Sprint PCR Thermocycler. As a control for RT-PCR product production, human actin expression was tested in RNA extracted from HeLa cells which was provided in a kit purchased from Invitrogen (in the reaction mixture, no $MgSO_4$ was included, the difference compensated for by 3 µl of DEPC treated water).

Primers

All primer sequences were obtained from the National Center Biotechnology Information (NCBI) website: http://www.ncbi.nlm.nih.gov/nuccore/NM_031966

They were designed to amplify 100-300 bp fragments and anneal between 58-60°C. For the purpose of demonstrating how all the proteins listed in Table 1 were evaluated, the procedure and results obtained in the evaluation of KI-67 are presented.

Actin

Forward primer sequence: 5' CAA ACA TGA TCT GGG TCA TCT TCT C 3'

Reverse primer sequence: 5' GCT CGT CGT CGA CAA CGG CTC

PCR product produced was 353 base pairs in length

Bcl-2

Forward primer sequence: 5' GAG ACA GCC AGG AGA AAT CA 3'

Reverse primer sequence: 5' CCT GTG GAT GAC TGA GTA CC 3'

PCR product produced was 127 base pairs in length.

KI-67

Forward primer sequence: 5' TTG GAG AAT GAC TCG TGA GC 3'

Reverse primer sequence: 5' CGA AGC TTT CAA TGA CAG GA 3'

PCR product produced was 218 base pairs in length.

Detection and Quantitation of Product

Agarose Gel Electrophoresis

1.5% agarose gels were prepared in a 50 ml volume of TBE buffer (1X solution: 0.089 M Tris borate and 0.002M EDTA, pH 8.3), containing 3 µl of ethidium bromide in a Fisher Biotest electrophoresis system. Samples were run for 2 hours at a constant voltage of 70 using a Bio-Rad 1000/500 power supply source. To locate the amplified PCR product, 3 µl of a molecular marker (Invitrogen) which contained a sequence of bases in 100 base pair increments (Invitrogen) as well as 2 µl of a sucrose based bromophenol blue tracking dye were run in each gel.

Quantitation

Gels were visualized under UV light and photographed using a Canon 800 digital camera. Photos were converted to black and white format and bands quantitated using Medical Image Processing and Visualization (Mipav) software provided by the National Institute of Health. Means and standard deviations were compared using Student *t*-tests to determine significance.

Results

Actin control and Bcl-2 Expression

As a control (Figure 1) for RT-PCR product produc-

tion, human actin expression was tested in RNA extracted from HeLa cells [1].

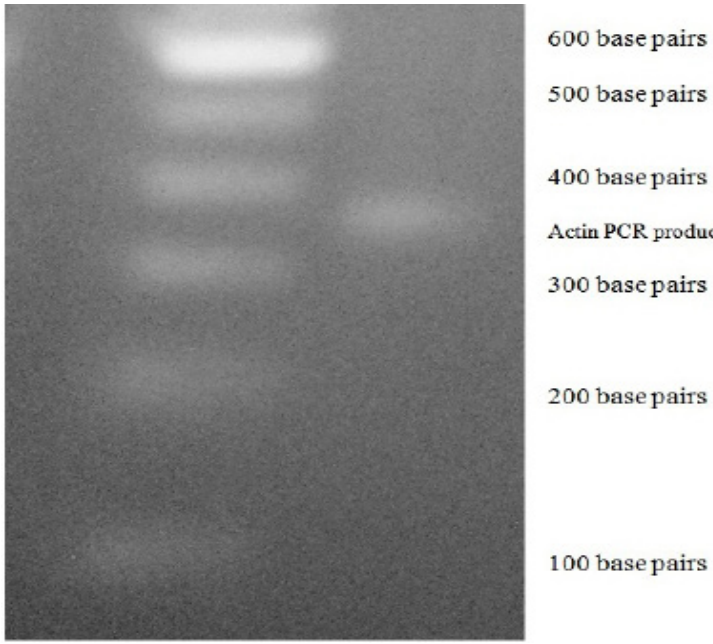

Figure 1: Human Actin Control in an Agarose Gel.
Actin 353 base pair product

To determine that the oligos had activity against bcl-2 they were similarly tested (Figure 2). LNCaP cells incubated for 24 hours in the presence of 6.25 μM of oligos suppressed bcl-2 expression, and support the finding of comparable biologic activity in both mono- and bispecific oligos measured in the *in vitro* cell growth inhibition experiments [1]. When photographs of the identified prod-

uct bands were scanned on agarose gels and quantitated using Mipav software, in a series of runs, the greatest expression of bcl-2 was always found in untreated LNCaP cells. Those treated with oligos, whether mono- or bispecific, produced bands which indicated obvious (to the naked eye) suppression. For each oligo evaluated, the greatest amount of suppression measured approached 100% for the mono-specific MR_4; and for the bispecific MR_{24} and MR_{42}, 86% and 100%, respectively. Suppression was found in both repeat PCR runs with bcl-2 primers, as well as in repetitive agarose gel quantifications [16]. A representative band is presented in Figure 2.

Figure 2: Bcl-2 Expression is Suppressed by Oligos as Indicated in a Representative Agarose Gel.

KI-67 Expression

Identical amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against bcl-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against KI-67. When background intensity was subtracted, the rela-

tive intensity of all bands corresponding to KI-67 representing cells treated with MR_4, MR_{24} and MR_{42} compared to controls were respectively increased 363.0% ± 158.9 (P = 0.000229), 427.1% ± 232.3 (P = 0.001137) and 355.7% ± 210.5 (P = 0.002017). These results were pooled from six duplicate gels and the statistically significant increased expression found in each type of oligo are among the greatest levels of relative increase seen in all previously reported experiments [1,5-7,8-12,15-18]. A representative gel is found in Figure 3.

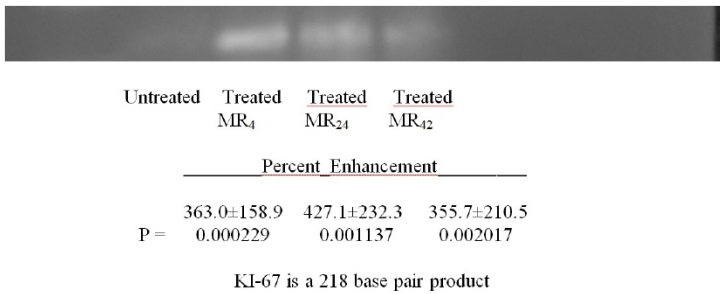

Figure 3: KI-67 Expression is Greatly Enhanced by Oligo Treatment as Indicated in a Representative Agarose Gel.

Table 1 summarizes the results obtained from our studies. KI-67 protein is the most enhanced in its expression, suggesting that increased proliferation, is the overall result of this type of gene therapy directed towards bcl-2 and restoration of apoptosis. This proliferation is the result of many compensatory mechanisms listed in the table.

Discussion

Gene therapy is often promoted as a highly specific and deliverable treatment to control aberrant gene expression by tumor cells (particularly when growth factors, their receptors or apoptosis inhibitors are excessively produced). However, it's now apparent that it's not as specific as previously thought. Antisense oligos consist of nucleotide bases synthesized complimentary in sequence to mRNA. When hybridized to mRNA, they produce a translational arrest of the targeted gene's mRNA expression. Now in clinical trials against a variety of solid tumors, this method is an effective, relatively non-toxic and inexpensive form of therapy and various types of antisense RNA have been constructed for this purpose. These include the phosphorothioated oligos used in these evaluations and other formulation including 2'-MOE-RNA, morpholinos, siRNA, miRNA etc. Modifications to the oligo backbone and base structure are used to prevent nuclease degradation, increase systemic half- life or enhance distribution and delivery. Some of these derivatives have been evaluated clinically, but all are directed against single gene transcriptional (mRNA) products. In contrast, the oligos discussed in this paper included both mono- and bispecific forms, each having a base sequence complementary to and directed against mRNA encoding the apoptosis inhibitor bcl-2, (bispecifics included an additional site directed against epidermal growth factor receptor [EGFR]). We evaluate bispecific oligos because it would be naïve to

believe targeting a single mRNA would be sufficient to produce a clinical response in most tumors, and activity at one site does not affect binding at a second [16], therefore administration of a single oligo having two mRNA targets could have an additional suppressive benefit. Furthermore, we have shown that both mono- and bispecific oligos have comparable activity suppressing bcl-2 [16].

While it's understandable that genes which share base sequence homology would also be susceptible to antisense oligos, when directed at common sequences, what is not expected are the effects on non-targeted genes, many of which control additional growth regulating pathways. We have also shown that certain complementary stretches of base sequences within the oligo could also produce unanticipated effects on the expression of cell surface antigens (and differentiation proteins). In an early evaluation of bispecifics we reported the enhanced expression of prostate specific membrane antigen (PSMA) [17] when oligos were directed against bcl-2. The unique capacity to produce such changes by these bispecfics (and not a similarly directed monospecific) is attributable to an unusual double strand conformation present in bispecifics and interferon induction (an enhancer of surface antigen expression) [18]. Such expression could enable better recognition and targeting by cytotoxic T cells [18].

Tumors are a mass of genetically unstable heterogeneous cells capable of both rapid mutation and selection. Just as bacteria and viral agents develop resistance to chemotherapeutics, tumors cells have a similar capability.

In prostate cancer it's thought that hundreds of genes (including those listed here) drive tumor cells to grow, in addition to the effects of androgen acting through the AR (as a transcriptional factor), AR coactivating proteins (p300, IL4, IL6), regulators of apoptosis (bcl-2, clusterin, AKT-1), transcriptional factors (MED-12, STAT-3) and various autocrine loops (involving transforming growth factor-alpha [TGF-α], its EGFR binding site, insulin like growth factor [IGF1] and its receptor [IGF-1R]).

Initial evaluation of protein expression associated with compensation regulating the traditional mode of apoptosis (mitochondrial mediated) focused on the bcl-2, bax, bad, clusterin etc. However, more recent work evaluated proteins associated with tumor cell destruction, via apoptosis, mediated by a secondary route for activation, involving direct signal transduction. This is a process of initiating apoptosis through the binding of activating proteins (ligands) to cell surface receptors. When ligands bind to these receptors they activate a destructive cascade of protein interactions which lead to cell death. These receptors are structurally similar to the tumor necrosis factor receptor (CD95) and also regulate the immune system's cytotoxic T cell response. As mediators of the immune system their expression can have unanticipated effects on certain types of therapy since these proteins, particularly PD-1 and PD-L1, are now being targeted by monoclonal antibodies to treat cancer patients via immune checkpoint

blockade, particularly those with melanoma, mesothelioma and lung cancer [19]. Our studies found that PD-1 its ligand PD-L1 and fas-ligand, were all significantly enhanced following bcl-2 suppression and therefore include immunoregulation, as an additional pathway for compensatory based resistance, to control of apoptosis [9].

Oligos (produced by Oncogenex Pharmaceuticals) have reached clinical trials for the treatment of prostate cancer (OGX-011), while others remain in preclinical development (OGX-225). Often administered in combination with traditional chemotherapy, these oligos target bcl-2, clusterin (OGX-011 in Phase II testing), heat shock protein 27 (OGX-427) or insulin growth factor binding proteins (OGX-225) [20]. Genta conducted a phase 3 test using oligos (Genasense; oblimersen) directed against bcl-2 for treating melanoma, chronic lymphocytic leukemia and various solid tumors [21], but compensatory effects produced by this agent were not reported. Many represent efforts to restore tumor apoptosis by eliminating suppressive bcl-2 [2-4] associated with treatment resistance. Similar approaches are directed at clusterin, but compensatory mechanisms activated by these oligos have not been evaluated. Since derivatives of antisense oligos (siRNA, miRNA) continue to be developed and tested, and while some directed against bcl-2 are in clinical trials, it is important to identify compensatory changes that result.

Bcl-2 and its related proteins continue to be target-

ed by new technologies. Small, specific molecules like DARPins (designated ankyrin repeat proteins) are genetically engineered antibody-like binding proteins, derived from natural ankyrin proteins (which are a common type of protein involved in cell signaling, regulation and structure). Some have reached preclinical trial development, and some target bcl-2. Should they reach clinical trial status, they too may produce the type of compensatory mechanisms summarized here, and potentially produce more aggressive tumor activity.

This year (2017) the American Cancer Society (ACS) estimated that in spite of early detection, screening for prostate specific antigen (PSA) and effective treatments for localized disease, in the United States there are 26,730 expected deaths from prostate cancer with 161,360 newly diagnosed cases [22]. New types of treatment, including gene therapy and translational inhibition must be developed and employed (probably in combination with traditional androgen ablation).

Conclusions

These results demonstrate that changes in expression resulting from suppression of bcl-2 involve compensation of those genes associated with apoptosis, androgen sensitivity, immune recognition, oncogenes and overall proliferation (possibly as a result). The implication is that such therapy could result in more aggressive tumor cell selection.

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