Is Immune Checkpoint Blockade Altered By Specific Gene Therapy Directed Against Bcl-2?

Author Details:
(1) **Marvin Rubenstein, Ph.D.** Courtney M. P. Hollowell, M.D. Patrick Guinan, M.D.

Division of Cellular Biology, Hektoen Institute for Medical Research (M.R.,P.G.); the Division of Urology, Stroger Hospital of Cook County (M.R., C.M.P.H., P.G.); the Departments of Biochemistry (M.R.) and Urology (M.R., P.G.), Rush University Medical Center; Department of Urology (P.G.), University of Illinois at Chicago, Chicago, IL, 60612.

Reprints and correspondence should be directed to: Dr. Marvin Rubenstein, Chairman-Division of Cellular Biology, Hektoen Institute for Medical Research, 2240 West Ogden Avenue, 2'nd floor, Chicago, IL 60612. Phone: 312-864-4621; Fax: 312-768-6010; email: DrMarv@Prodigy.net

Running title: Immune recognition altered in compensation for bcl-2 suppression

ABSTRACT

Antisense oligonucleotides (oligos) [1,2] have been administered against prostatic LNCaP cells in both in vivo and in vitro models. In spite of advances in early detection, the treatment of prostate cancer has not improved and it is estimated that 233,000 new cases will be diagnosed and 29,450 men will die from it this year in the United States [3]. Gene therapy and immune checkpoint blockade could provide some improvement. However, our studies indicate that gene therapy employing oligos directed towards bcl-2 (in LNCaP cells) frequently are compensated for by altered regulation of apoptosis, increased androgen sensitivity and enhanced oncogene activity [4]. In addition, we found that certain oligo conformations induce interferon [5], enhance cell surface antigen expression [prostate specific membrane antigen (PSMA)] [6] and potentially increase tumor recognition and targeting by the immune system.

We hypothesized that immunologic recognition is an additional pathway for compensation which follows suppressive bcl-2 treatment and suggest that this type of gene therapy could influence proteins associated with immune checkpoint blockade [7,8] now the "standard for care" treatment of melanoma and currently being evaluated for kidney and other solid tumors (including the prostate). Therefore, markers targeted by these new agents should be evaluated to identify changes in expression produced by previous therapy.

Key words: Antisense; Prostate cancer; Therapy

INTRODUCTION and BACKGROUND

Prostate cancer is similar to melanoma in several respects. Each can remain inactive for long periods of time, are frequently treated by irradiation and express tumor associated antigens (gp100/melanoma and PSA, PSMA, PAP and PCA-3/ prostate). Irradiated tumors shed antigens and provide recognition targets for activated T cells (unless inhibited by T_{reg} suppressor cells). Immune checkpoint blockade works by several mechanisms which include targeting suppressive proteins (like CTLA-4) which interferes with the stimulatory interaction of T lymphocyte CD28 with B7 on antigen presenting cells. The result is continued anti-tumor immunologic responsiveness and prevention of tumor induced anergy and T cell tolerance. Anti-CTLA antibodies also expand T_{reg} cells in lymph nodes, but cause their depletion in the tumor microenvironment [8]. Such therapy could be employed following or in combination with gene therapy (including the use of antisense oligos). In addition to targeting the inhibitory signaling of T-cell CTLA-4, newer agents (monoclonals) have targeted the programmed death protein (PD-1) expressed by tumors, and its ligand (PD-L1). When expressed, PD-1 acts as a tumor expressed cloacking device, inhibiting specific cytotoxic lymphoid activity. If immune blockade therapy were to enter clinical trials for prostate cancer, these targets could be influenced by prior gene therapy and affect outcomes, therefore changes in expression must be taken into account.

Antisense oligonucleotides (oligos) have been employed in both *in vivo* and *in vitro* prostate cancer models employing LNCaP and PC-3 cell lines. Genes targeted include protein growth factors, androgens, receptors for stimulating factors, inhibitors of apoptosis (BCL-2 and clusterin) and oncogenes. Some (developed by Oncogenex Pharmaceuticals) have entered clinical trials (OGX-011) while others are in preclinical development (OGX-225) [9]. Oligos act through a variety of mechanisms and provide a specific and relatively non-toxic method for translational arrest by degradation of annealed mRNA:oligo duplexes by RNase H [10], protein binding, DNA triplex formation [11,12] and especially DNA-RNA triplex formation [13]. In attempts to increase the efficacy of oligos our lab has been evaluating bispecific derivatives [14-20] with more than one binding site on a single DNA strand. Bispecifics were developed in order to suppress the expression of two gene products. In LNCaP cells we found that mono-specific oligos targeting bcl-2 and bispecifics targeting both bcl-2 and the epidermal growth factor receptor (EGFR) have comparable activities in suppressing BCL-2 expression as

determined by both *in vitro* growth and BCL-2 expression [20]. Therefore, the presence of a second binding site does not diminish activity of the other.

In theory gene therapy should be specific and lack non-specific effects. In practice this is not so, and the non-specific effects can compromise the initial attempt to control tumor growth. Since these approaches are now in clinical practice, the recognition of side effects is increasingly important to enhance efficacy of this innovative therapy. In the LNCaP model, cells treated with mono- or bispecific oligos targeting bcl-2 were extracted for RNA and evaluated protein expression employing RT-PCR. We found that inhibition of BCL-2 with antisense oligos suppressed the apoptotic promoter caspase-3 [19] and enhanced expression of the androgen receptor (AR) [22], p300 [22], interleukin-6 (IL-6) [22], oncogenes viral myelocytomatosis (v-MYC) [22] and signal transducer and activator of transcription3 (STAT3) proteins. This suggested that following BCL-2 suppressive therapy, there could be selective pressure for a more aggressive (androgen-sensitive, oncogene driven) phenotype. Recognition of this transition is important if either gene therapy or immune checkpoint blockade were to be employed.

THE HYPOTHESES

We hypothesized that immune regulation could provide an additional pathway to evade therapy through compensation. To test this theory we once again used RT-PCR to evaluate the immune regulatory markers for programmed death-1 (PD-1) and its ligand (PD-1L) and found significant enhancement of both. However, enhancement of PD-L1, although significant is relatively small, suggesting that in this system therapy directed against PD-L1 could be more effective following suppressive BCL-2 therapy than that directed against PD-1. PD-1 is a protein which is tumor cell associated and interacts with T cells in a manner which induces tumor tolerance. If immune checkpoint blockade treatment were to be used against prostate cancer it is important to evaluate the suitability of each target, whether it be protein or ligand and how expression may be altered by prior treatment. A variety of monoclonals directed against either PD-1 or PD-L1 are in commercial development, and several (MK-3475 and nivolumab) are in the FDA approval process.

To more fully understand the effects of prior treatment on subsequent treatment based on immune blockade, we proposed that additional immune-regulators be similarly evaluated, to include fas, interferon, interleukin-2, tumor necrosis factor and their respective activating ligands and receptors. These proteins regulate several pathways leading towards cell death and immune potentiation and could provide an overall view of the compensatory non-specific effects of therapy.

EVALUATION OF HYPOTHESES

Oligonucleotides

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

Base Sequences

Each oligo contains at least one CAT sequence and target the area adjacent to the mRNA AUG initiation codon for the 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 was either supplemented or replaced with fresh.

Determination of Growth

Four days prior to the addition of oligos 1 X 10^4 LNCaP cells were added, in a total 200 µl volume of media, to each depression of a 96 well plate and incubated at 37° C in a 5% CO_2 incubator. On the day of transfection the following solutions were prepared:

A) 1 µl of buffer containing either oligo or a diluent is added to 50 µl of OPTI-MEM and gently mixed.

B) 1 µl of Lipofectin is diluted in 50 µl of OPTI-MEM and mixed gently for 5 minutes at room temperature.

C) Oligo dilutions are added to 50 µl of Lipofectin and gently mixed for 20 minutes at room temperature.

D) 100 µl of the Lipofectin and oligos are added to 100 µl of RPMI medium and mixed.

Cells were incubated for 24-48 hrs before solutions are aspirated and re-incubated for an additional 48 hrs in 200 µl of media. Cell counts were determined following the addition of WST-1 reagent to each well, and after 2 hrs the color intensity was measured by a micro-plate reader at a wavelength of 450 nm, using a reference of 650 nm. Values obtained were determined after the subtraction of paired blank samples from the

experimental wells and multiplied by a constant to give whole integers for analysis. Microsoft Excel software was utilized to calculate means and standard deviations and Students t tests are used to determine significance.

Oligo Treatment Prior to PCR

Fours days prior to oligo addition, when cell density approaches 75% confluence, 10 ml of fresh media was added. Cells were then incubated for an additional 3 days before 5 ml of media was replaced with fresh the day before oligos were to be added. 100 µl of stock oligos was 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.

RNA Extraction

Following treatment, media was removed, a single ml of cold (4°C) RNAzol B 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 remained 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 then 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 then 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 florometer and Quant-iT RNA assay kit (invitrogen). DEPC is an inhibitor of RNase activity.

RT-PCR

Extracted RNA was diluted to 40 µg/µl in DEPC treated water and 1-4 µl of this RNA added to1 µl of both sense and antisense primers (forward and reverse sequences). From a kit available 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 $MgSO_4$ (3 µl of a stock concentration of 5mM, used for bcl-2 vials only). DEPC treated water was added to yield a final volume of 50 µl. As a control for RT-PCR product production, human actin expression was tested in RNA extracted from HeLa cells which is provided in a kit also available from invitrogen. RT-PCR is performed for 2 X 25 cycles using the F54 program in a Sprint PCR Thermocycler

Primers:

Primers for fas, Interferon, Interleukin-2, tumor necrosis factor and their respective ligands and receptors were purchased from RealTimePrimers (Elkins Park, PA). The primer sequences for our preliminary work which is summarized here are listed below.

PD-1

Forward primer sequence: 5' GAC TAT GGG GAG CTG GAT TT 3'

Reverse primer sequence: 5' AGA GCA GTG TCC ATC CTC AG 3'

PCR product produced was 192 base pairs in length.

PD-L1

Forward primer sequence: 5' TGA TAC ACA TTT GGA GGA GAC G 3'

Reverse primer sequence: 5' CCC TCA GGC ATT TGA AAG TAT C 3'

PCR product produced was 261 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 BioRad 1000/500 power supply source. To locate the amplified PCR product, 3 µl of a molecular marker (Invitrogen) which contains a sequence of bases in 100 base pair increments (Invitrogen) as well as 2 µl of a sucrose based bromphenol blue tracking dye were run in each gel. For actin product localization, the tracking dye is included in each sample run; for all others the tracking dye ran separately.

Quantitation

Gels were visualized under UV light and photographed using a Canon PowerShot ELPH 300HS digital camera. Photos were converted to black and white format and bands quantitated using Mipav software provided by NIH.

RESULTS

Preliminary Findings

Cell Culture Experiments

The equivalence of mono- and bi-specific oligos directed against bcl-2 has been established and previously published [18]. LNCaP cells were incubated with MR_4, MR_{24} and MR_{42} and compared to lipofectin containing controls. In an initial experiment each oligo significantly inhibited the growth of LNCaP cells: MR_4 by 23.8% ($p = 0.0004$); MR_{24} by 31.2% ($p < 0.001$); and MR_{42} by 31.7% ($p < 0.001$).

In a repeat experiment LNCaP cells were similarly incubated and compared to lipofectin containing controls. Bispecific oligos MR_{24}, and MR_{42} produced significant respective inhibitions of 49.5% (p <

0.001) and 56.8% ($p < 0.001$), and were at least as effective as the mono-specific MR_4 directed only towards bcl-2 in the inhibition of *in vitro* cell growth.

RT-PCR Experiments

As a control, comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against bcl-2 (and EGFR in the bispecifics). In a series of control experiments (data not shown) to validate RNA extraction and RT-PCR procedures, the expression of human actin in HeLa cells was identified [19].

PD-1 Expression

Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against BCL-2 (and EGFR in the bispecifics) was then evaluated by RT-PCR using primers directed against PD-1. A representative band is presented in Figure 1.

When background intensity was subtracted, the relative intensity of the bands corresponding to PD-L1 representing cells treated with MR_4, MR_{24} and MR_{42} compared to controls were increased 149.3% ± 113.3 (P = 0.018476), 320.7% ±281.0 (P = 0.034078), and 193.9% ± 117.0. (P = 0.005988) (mean ± SD). These results indicate that the expression of the immune blockade marker PD-1 is significantly enhanced suggesting once again another compensatory response to BCL-2 suppression may occur.

PD-L1 Expression

Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against BCL-2 (and EGFR in the bispecifics) was then evaluated by RT-PCR using primers directed against PD-L1. A representative band is presented in Figure 2.

When background intensity was subtracted, the relative intensity of the bands corresponding to PD-L1 representing cells treated with MR_4, MR_{24} and MR_{42} compared to controls were increased 33.3% ± 20.1 (P = 0.006079), 51.0% ±36.7 (P = 0.014394), and 28.5% ± 19.3 (P = 0.010793) (mean ± SD). These results indicate that the expression of the immune blockade marker PD-L1 is significantly enhanced (to lesser extent), suggesting once again another compensatory response to BCL-2 suppression may occur.

EVALUATION OF HYPOTHESIS

Similar experiments to those presented above would be performed employing primers for both forward and reverse sequences of genes associated with death, growth, differentiation and immune regulation. This would include, but not necessarily limited to, proteins fas, interferon, interleukin-2 and tumor necrosis factor, in addition to their respective receptors and ligands.

DISCUSSION

Although great strides have been made for the treatment of cancer using the triad of surgery, radiation and chemotherapy, this group of diseases is estimated to afflict an estimated 1,665,140 Americans and kill 585,720 [3], in 2014. Although continued improvements can be made in these therapies, new approaches are necessary to be employed either alone or in some type of combination with the above. Advances in molecular biology and immunology have led to new types of treatment, focusing on either the individual or the immune system. These include gene therapy with oligos, targeted therapy based upon individual tumor genetic profiles (BRAF mutations in melanoma), cellular gene therapy (using vectors to transfect genes or oligos) particularly those directed to regulate the immune system and immune checkpoint blockade [23,24,25]. Each of these treatments continue to evolve, based upon the discovery and characterization of new protein mutations and immune checkpoint regulators.

The future will include a variety of such agents being used, either alone or in combination. While chemotherapy and some targeted treatments often lead to tumor resistance, immune checkpoint blockade enhances the activity of the immune system, and should not itself make tumors resistant. Instead, tumors remain susceptible to an activated specific immunity and these agents retain suitability for re-administration either as a periodic immune stimulus or following recurrence. The discovery of numerous immune checkpoint proteins and therapy directed at more than one (either alone or in combination) make this an exciting field, where advances develop rapidly and, pending FDA approval, new agents become available quickly.

At this time it is necessary to emphasize that some types of therapy, particularly gene directed, can alter some of the targets of immune checkpoint blockade therapy. However, due to the development of multiple agents by various companies, this can be overcome by employing one of the alternative agents targeting either the same protein, or pathway. Therefore, gene expression changes that follow drug administration in efforts for the tumor to compensate must be evaluated. If changes are noted, the therapy chosen should take these into account. As an example, we evaluated both PD-1 and PD-L1 expression following bcl-2 suppressive therapy. Although both proteins increased significantly, the enhancement of PD-L1 was not as great. Therefore, this could make it a better target for monoclonal antibodies than PD-1. It is imperative to understand the consequences of initial therapy and possible adverse effects produced later. Failure to recognize these compensatory mechanisms could lead to the activation of genes associated with a more aggressive, hormone sensitive, oncogene driven stage, as suggested in this

LNCaP model. Evaluation of fas, interferon, interleukin-2, tumor necrosis factor and their respective receptors and ligands are only some of the immunoregulatory proteins which must be evaluated. In addition to oligonucleotides which regulate expression of microRNAs [26] should be similarly studied. As hypothesized, many others are probably involved and subject to tumor compensatory mechanisms, particularly (as previously indicated) those involving apoptosis, hormone sensitivity and oncogene activity.

CONCLUSION

We hypothesized and now conclude that immunologic recognition is an additional pathway for compensation which follows suppressive bcl-2 treatment and suggest that this type of suppressive gene therapy, particularly that mediated by oligonucleotides (and in this case directed against BCL-2) could influence proteins associated with immune checkpoint blockade, altering efficacy.

Acknowledgments:

The Cellular Biology laboratory at the Hektoen Institute is supported, in part, by the Blum Kovler Foundation, the Cancer Federation, Lawn Manor Beth Jacob Hebrew Congregation, the Max Goldenberg Foundation, the Pritzker Taubert Family Foundation, the Safeway/Dominicks Campaign for Breast Cancer Awareness, the Sternfeld Family Foundation, and the Herbert C. Wenske Foundation.

REFERENCES

1) Rubenstein JL, Nicolas JF, Jacob F [1984] L'ARN non sens (nsARN): Un outil pour inactiver specifique mentl'expression d'un gene donne in Vivo [Nonsense RNA: A tool for specifically inhibiting the expression of a gene in Vivo]. Comptes Rendus Academie des Sciences Paris 299:271-4.
2) Weintraub H, Izant JG, Harland RM [1985] Anti-sense RNA as a molecular tool for genetic analysis. Trends in Genetics 1:22-25.
3) Siegel R, Ma J, Zou Z, Jemal A [2014] Cancer statistics, 2014. CA Cancer J Clin 64:9-29.
4) Rubenstein M, Hollowell CMP, Guinan P [2013] No compensation in CD44 stem cell marker following bcl-2 suppression by antisense oligonucleotides. In Vivo 27:251-6.
5) Rubenstein M, Hollowell CMP, Guinan P [2011] Bispecific oligonucleotides may induce interferon in LNCaP cells enhancing surface antigen expression: Effect of intra strand base pair complementarity. In Vivo 25:61-7.
6) Rubenstein M, Hollowell CMP, Guinan P [2012] Differentiated prostatic antigen expression in LNCaP cells following treatment with bispecific antisense oligonucleotides directed against bcl-2 and EGFR. Med Oncol 29:835-41.
7) Pardoll DM [2012] The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12:252-64.
8) Allison J [2013] Immune checkpoint blockade in cancer therapy: New insights and opportunities. Mol Cancer Ther 12: PL07a.
9) www.oncogenex.ca/
10) Walder RY, Walder JA [1988] Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. Proc Natl Acad Sci USA. 85:5011- 5015.
11) Felsenfeld G, Miles HT [1957] Formation of a three-stranded polynucleotide molecule. J Amer Chem Soc 79:2023-4.
12) Durland RH, Kessler DJ, Hogan M. Antiparallel triplex formation at physiological pH. In: Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS. E. Wickstrom (Ed.), Wiley-Liss, New York, 1991, 219-226.
13) Hélène C [1994] Control of oncogene expression by antisense nucleic acids. Eur J Cancer 30:1721-6.
14) Rubenstein M, Tsui P, Guinan P [2006] Bispecific antisense oligonucleotides with multiple binding sites for the treatment of prostate tumors and their applicability to combination therapy. Methods Find Clin Pharmacol 28:515-8.
15) Rubenstein M, Tsui P, Guinan P [2007] Combination chemotherapy employing bispecific antisense oligonucleotides having binding sites directed against an autocrine regulated growth pathway and bcl-2 for the treatment of prostate cancer. Med Oncol 24:372-8.
16) Rubenstein, M., Tsui, P., Guinan, P [2009] Multigene targeting of signal transduction pathways for the treatment of breast and prostate tumors. Comparisons between combination therapies employing bispecific oligonucleotides with either Rapamycin or Paclitaxel. Med Oncol 26:124-30.
17) Rubenstein M, Tsui P, Guinan P [2005] Construction of a bispecific antisense oligonucleotide containing multiple binding sites for the treatment of hormone insensitive prostate tumors. Med Hypotheses 65:905-7.
18) Rubenstein M, Tsui P, Guinan P [2007] Bispecific antisense oligonucleotides having

binding sites directed against an autocrine regulated growth pathway and bcl-2 for the treatment of prostate tumors. Med Oncol 24:189-96.

19) Rubenstein M, Tsui P, Guinan P [2008] Treatment of MCF-7 breast cancer cells employing mono- and bispecific antisense oligonucleotides having binding specificity towards proteins associated with autocrine regulated growth and bcl-2. Med Oncol 25: 182-6.

20) Rubenstein, M., Guinan, P 2010] Bispecific antisense oligonucleotides have activity comparable to monospecifics in inhibiting expression of bcl-2 in LNCaP cells. In Vivo 24:489-93.

21) Rubenstein M, Hollowell CMP, Guinan P [2011] Inhibition of bcl-2 by antisense oligonucleotides is followed by a compensatory suppression of caspase-3 in LNCaP cells. Eur J Clin Med and Oncol 3:1-61.

22) Rubenstein M, Hollowell CMP, Guinan P [2013] Increased expression of the androgen receptor with p300 and IL-6 coactivators compensate for oligonucleotide suppression of bcl-2. No increased CREB binding protein or IL-4 expression. Ther Adv Urol 5:85-93.

23) Schlingensiepen K-H, Fischer-Blass B, Schmaus S, Ludwig S [2008] Antisense therapeutics for tumor treatment: The TFF-beta2 inhibitor AP12009 in clinical development against malignant tumors. Recent Results in Cancer Res 177:137- 50.

24) Hau p, Jachimczak P, Bogdahn U [2009] Treatment of malignanat gliomas with TGF-β2 antisense oligonucleotides. Expert Review of Anticancer Therapy 9:1663-74.

25) Trojan J, Ly A, Wei MX, Bierwagen M, Kopinski P, Pan Y, Ardourel M-Y, Dufour T, Shevelev A, Trojan LA, Francois J-C, Andres C, Popiela T, Chatel M, Kaspirzak H, Anthony DD, Duc HT [2010] Antisense anti IGF-I cellular therapy of malignant tumours: Immune response in cancer patients. Biomedicine & Pharmacotherapy 64:576-8.

26) Shaked I, Meerson A, Wolf Y, Avni R, Greenberg D, Gilboa-Geffen A, Soreq H [2009] MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity 31:965-73.

FIGURE 1
Significantly Increased Expression of PD-1 in Representative Agarose Gel

Untreated	Treated MR_4	Treated MR_{24}	Treated MR_{42}

	Control	MR4	MR24	MR42
Mean	0	149.34	320.7	193.92
SD	0	113.2668	281.0184	116.9955

P vs control			
	Mono	Bispecific	Bispecific
	0.018476	0.034078	0.005988

PD-1 is a 192 base pair product

FIGURE 2

Significantly Increased Expression of PD-L1 in Representative Agarose Gel

Untreated	Treated MR_4	Treated MR_{24}	Treated MR_{42}

	Control	MR4	MR24	MR42
Mean	0	33.26	51.04	28.5
SD	0	20.12295	36.66965	19.28756

P vs control			
	Mono	Bispecific	Bispecific
	0.006079	0.014394	0.010793

PD-L1 is a 261 base pair product