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14. ABSTRACT Protocadherin-PC (PCDH-PC or PCDH11Y) is a human-, male-specific gene product that is upregulated in prostate cancer cells by androgen deprivation therapy. Silencing of this gene product with PCDH-PC-specific siRNA drastically induced the death of prostate cancer cells cultured in the absence of androgens and we have proposed that PCDH-PC expression knockout by shRNAs or antisense oligonucleotides (ASOs) might be useful in preventing the development of castration-recurrent prostate cancer in prostate cancer patients. To this end, we have created functional shRNA vectors and ASOs capable of suppressing PCDH-PC expression and we have also created a monoclonal antibody that allows us to detect PCDH-PC protein in cell extracts or tissues. The antibody preferentially recognizes prostate cancer cells in human prostate specimens. Knockdown of PCDH-PC expression by the shRNA vectors is more efficient than with siRNA and is apparently sufficient to kill prostate cancer cells even under conditions where androgen is available and this further supports the idea that PCDH-PC contributes to the biological properties and survival of prostate cancer cells.					
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Annual Progress Report (III)

DOD Project: DAAMRDC W81XWH-06-00

Title: *Targeted Elimination of PCDH-PC Expression for Control of Hormone-Resistant Prostate Cancer*

Principal Investigator: Ralph Buttyan, Ph.D.

Progress Period: November 2007 to November 2008

Introduction: Protocadherin-PC (PCDH-PC) is an androgen-repressed gene encoded on the human Y-chromosome that is over-expressed in prostate cancer cells that were selected for resistance to apoptosis (1). Transfection of this gene product back into prostate cancer cells induces apoptosis- and hormone-resistance (1,2). PCDH-PC is also highly upregulated in androgen-sensitive human prostate cancer cells when they are exposed to androgen-free conditions (1). In our preliminary studies, we found that siRNAs that target and suppress the expression of PCDH-PC were able to dramatically increase the number of prostate cancer cells killed by exposure to androgen-free conditions. Based upon these results, we proposed that agents that target and block PCDH-PC expression might be used in conjunction with hormonal therapy to increase response and survival of advanced (metastatic) prostate cancer patients. The work in this project will develop strategies to suppress PCDH-PC expression in prostate cancer cells based on shRNA and antisense oligonucleotide targeting. We will then test whether cultured human prostate cancer cells (LNCaP) that are crippled with regards to their ability to upregulate PCDH-PC (by transfection with a PCDH-PC specific targeting shRNA), are killed when they transferred to androgen-deprived medium and whether these cells are unable to form hormone-refractory tumors when xenografted into male immunodeficient mice that are subsequently castrated. We will also test whether the treatment of LNCaP-xenografted mice with antisense oligonucleotides that target PCDH-PC, prevent these mice from developing hormone refractory tumors after they are castrated. In summary, the work in this project will pre-clinically test the idea that PCDH-PC targeting strategies might be a useful adjuvant therapeutic when advanced prostate cancer patients undergo hormonal therapy.

Body: This project has 4 Specific Aims and progress will be discussed for each Aim.

Specific Aim 1. Design shRNA expression plasmids that suppress PCDH-PC expression in LNCaP cells and isolate transfected variants of the LNCaP cell line that are unable to induce PCDH-PC expression when cultured in androgen-free medium.

Work Done: In last years progress report, we described our success in obtaining and characterizing two shRNA vectors (#310598 and 310600) that effectively silence PCDH-PC expression by greater than 90% in cells (293T-T6 or LNCaP-T6) that overexpress this protein (Figure 1). When we attempted to transfect these shRNA vectors into parental LNCaP cells (that express low levels of PCDH-PC, we were not able to obtain any stable clones. In contrast, control (non-targeting) shRNA vectors resulted in abundant colonies (> 200/dish). Cells transfected with shRNA-targeting vectors #310598 and #310600 did not produce any surviving colonies. This experiment was repeated with similar results. These experiments are remarkable since the most effective PCDH-PC-targeting shRNAs (#310598 and #310600) do not support puromycin-resistant colony following transfection into LNCaP cells and this may be because they are suppressing the low level expression of this gene product in these cells.

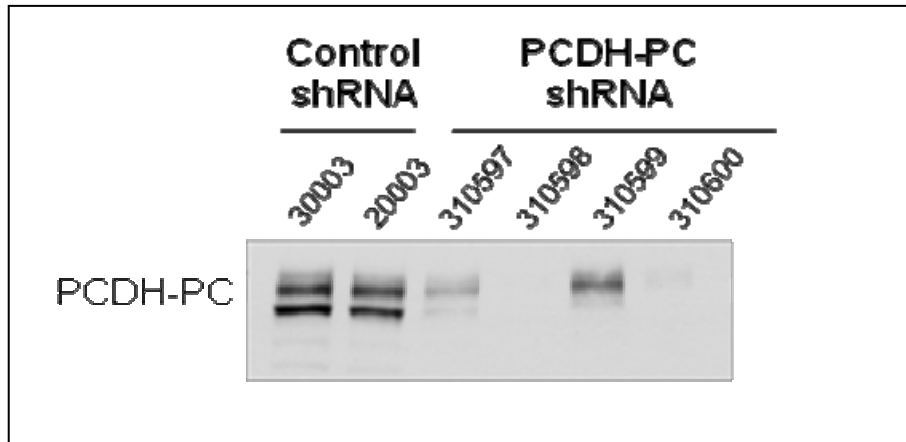


Figure 1 (Above). Reduction of PCDH-PC expression by shRNA targeting vectors. Western blot shows suppression of PCDH-PC expression in 293T cells by PCDH-PC targeting shRNA vectors (#310597-310600) when co-transfected along with a myc-tagged cDNA expression vector containing PCDH-PC cDNA. Cell extracts were prepared 48 hrs after co-transfection and were electrophoresed on an SDS-PAGE gel and blotted onto a filter. The filter was probed with an anti-myc antibody. The levels of myc-tagged PCDH-PC protein in cells co-transfected with control shRNA vectors (#30003 and 20003) is sharply contrasted with the effects of co-transfection with any of the PCDH-PC targeting vectors (#310597-310600). However the effects of two of the shRNA targeting vectors (#310598 and 310600) were especially pronounced with virtual complete suppression of PCDH-PC expression in these cells. These results were also reproduced in LNCaP cells.

Ongoing Work: We are currently attempting to transfect the two most effective PCDH-PC shRNA targeting vectors into PC-3 cells which do not have the potential for expressing PCDH-PC since these cells lack the human Y chromosome. If they we are able to efficiently obtain surviving clones with these cells, this will support the idea that the shRNA targeting PCDH-PC is lethal even for cells that express very low levels of this gene. Our results of transfection of these vectors into LNCaP cells suggests the need for a regulatable shRNA expression vector to complete the work proposed in Aim 1. To address this conundrum, we are constructing tetracycline-regulatable shRNA targeting vectors using the hairpin sequences in our most effective shRNA vectors and we believe that these vectors will enable us to complete the work proposed in Aim 1 as described.

Specific Aim 2. Design and test antisense oligonucleotides (ASOs) that suppress PCDH-PC expression in prostate cancer cells.

Work Done: We used the sequence of our most successful siRNA (#181, ref 2) to design simple antisense oligonucleotide (19 bases). Shown below in Figure 2 (below), are the results obtained when this ASO was applied to the medium of cells that were previously transfected with a PCDH-PC expression plasmid in which the PCDH-PC protein is “tagged” by a myc-peptide. Expression of the PCDH-PC protein is identified using an anti-myc antibody on Western blots. Results show that this ASO is effective in silencing PCDH-PC expression at an approximately 200 μ M concentration or greater. We believe that this data indicates that ASO #181 suitably targets PCDH-PC at concentrations above 200 μ M and completes the work described for Specific Aim 2.

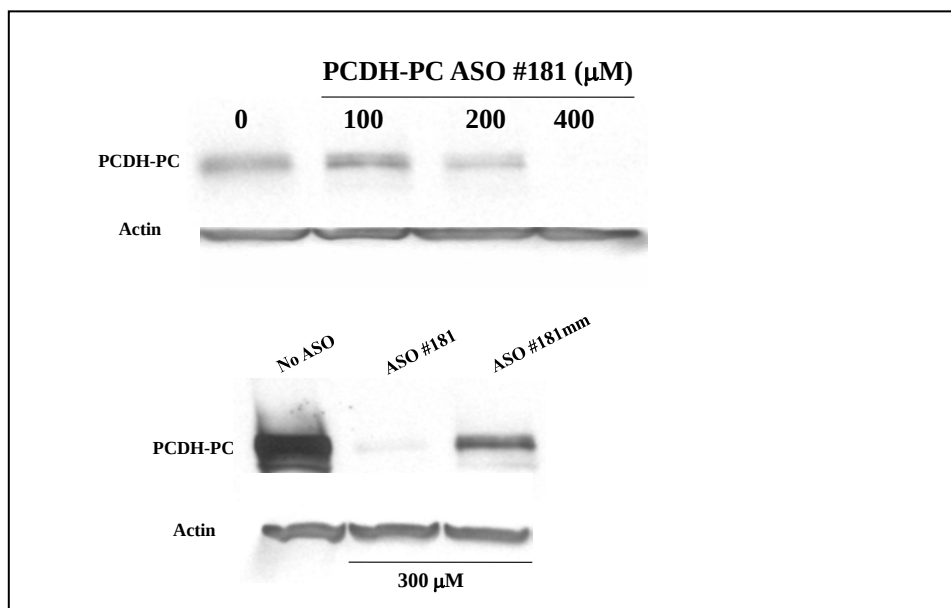


Figure 2 (Above). Reduction of PCDH-PC expression by ASO #181. (Top Panel). ASO #181 was transfected into LNCaP cells 24 hrs subsequent to transfection with a myc-tagged PCDH-PC expression vector. 48 hrs later extracts were made of the cells and the extracts were analyzed by Western blot for expression of myc-tagged PCDH-PC protein (110 kd). Results show significant reduction of PCDH-PC expression at 200 uM and higher concentration. (Bottom Panel) The experiment was repeated at a 300 uM ASO concentration and compared to the same concentration of the 181-mm ASO (containing 4 mismatched bases). Results show significant specificity to ASO 181-mediated reduction of PCDH-PC expression.

Specific Aim 3. Test PCDH-PC expression reduction strategies (shRNA or ASO targeting) for ability to induce death of LNCaP cells *in vitro* in androgen-free medium.

Work Done: As was indicated in the discussion of our results under Specific Aim 1, our effective PCDH-PC shRNA targeting vectors appear to be lethal for prostate cancer cells even when grown in medium (standard RPMI + 10% FBS) that contains very low levels of androgen. Our results indicate the possibility that even the low levels of PCDH-PC expressed in LNCaP cells cultured under these conditions is important for their survival. Therefore, we are developing a tetracycline-repressible shRNA targeting vector that will allow us to better control the expression of the PCDH-PC targeting shRNAs in LNCaP cells and adjust the PCDH-PC suppression so that we can complete this experiment as described.

Ongoing Work: We are constructing a tetracycline-inducible PCDH-PC targeting shRNA vector that will enable us to modulate the suppression of PCDH-PC expression in prostate cancer cells and complete the Aim as described.

Specific Aim 4 Proof-of-principle and pre-clinical testing of targeted PCDH-PC expression knockout combined with androgen deprivation as a strategy for prostate cancer therapy using mouse xenograft tumor models.

No work was done on this Specific Aim during the last period. This work will be carried out upon derivation of cells with conditionally-inducible shRNA.

New Work Accomplished Not Present in Original Specific Aims

Additional work was also undertaken outside of the original Specific Aims. Though not specified in the original Specific Aims, we feel that this work is relevant to the outcome of this project. This work includes:

- 1) *Development of a new monoclonal antibody that recognizes the PCDH-PC protein and preliminary use of this antibody for immunostaining of human prostate cancer tissue microarrays.* We contracted with a company (Promab, Inc., Albany, CA) to produce a mouse monoclonal antibody against a peptide that is exclusively present in the open reading frame of the PCDH11 expressed male homologue (PCDH_Y/PCDH-PC). We identified two different monoclonal cell populations that produced an antibody that recognized the recombinant PCDH-PC protein produced by transfected 293T cells and these antibodies also recognize the low levels of endogenous protein produced by low-passage LNCaP cells on Western blots (see Figure 3, below) as well as the higher levels of PCDH-PC protein produced in high passage (more androgen-resistant) LNCaP cells. One of the antibodies (5F3C8) was used for preliminary immunostaining of a human tissue microarray (done by Cybrdi, Inc., Rockville, MD) containing human prostate tissue samples (2 each) from 40 different (unidentified) donors. The results showed that strong immunoreactivity was observed in the majority of the tumor tissues, especially in tumor cells. Some stromal cells also showed mild immunoreactivity. The staining tends to be intracellular of cytoplasm and nucleus. There was no positive staining noted in normal prostate tissues. The chi-square test identified that the cancer-specific staining was significant ($p = 0.032$), however, no significance was found with regards to the staining between low (I + II or high grade tumors (III + IV). In summary, the PCDH-PC antibody specifically recognizes prostate cancer. At this time, we are exploring a collaboration with Dr. Martin Gleave of The Prostate Centre in Vancouver to utilize this antibody for analysis of tissue microarrays containing prostate cancer specimens from hormone-naïve or hormone-treated patients.

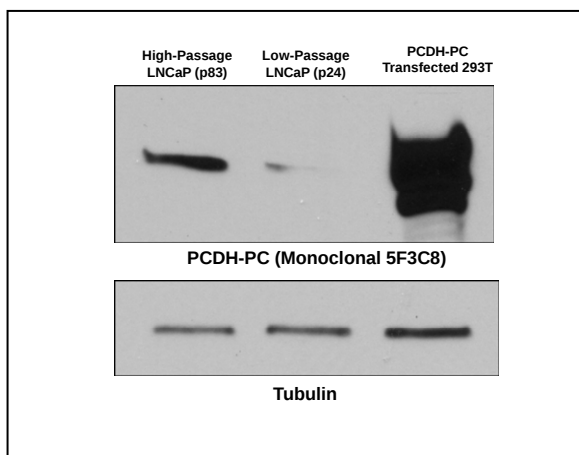
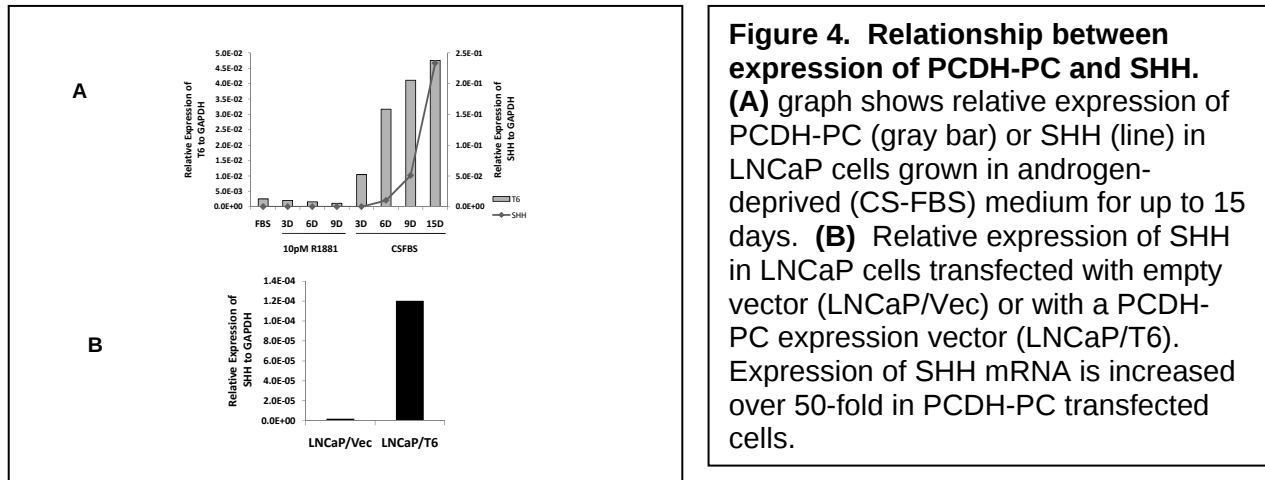


Figure 3. Western blot using new monoclonal antibody against PCDH-PC identifies recombinant PCDH-PC protein (right lane) as well as endogenous PCDH-PC protein expressed in high passage (left lane) or low passage (right lane) LNCaP cells.

- 2) *Relationship between the expression of PCDH-PC and expression of Sonic Hedgehog in prostate cancer cells.* PCDH-PC expression is highly upregulated by androgen deprivation. During the last period, we have identified another gene product, Sonic Hedgehog (SHH) that is upregulated coordinately with PCDH-PC in androgen-deprived LNCaP cells. SHH is an embryogenic tissue growth factor and morphogen that mediates signaling through the

hedgehog pathway. There is extensive interest in the hedgehog pathway as a mediator of oncogenesis in several human tumor systems including prostate cancer. The graph in Figure 4A shows the relative expression (qPCR data indexed to GAPDH expression in the same specimen) of these two genes as a function of time after androgen withdrawal (CS-FBS). The kinetics of their induction is extremely similar. To further test for a potential relationship between these two gene products, we transfected LNCaP cells with a PCDH-PC expression vector and then compared expression of SHH 48 hrs later to cells that were transfected with a control (empty) vector. As is shown in Figure 4B, SHH expression is increased over 50-fold in the cells that were transfected with PCDH-PC. We are currently testing whether si/shRNA that targets PCDH-PC prevents the upregulation of SHH in androgen deprived medium.



Key Research Accomplishments

- Testing and identification of effective shRNA targeting vectors that suppress expression of PCDH-PC in prostate cancer cells
- Design and testing of an effective anti-sense oligonucleotide that suppresses expression of PCDH-PC in prostate cancer cells
- Demonstration that shRNAs targeting PCDH-PC expression in prostate cancer cells effectively kill these cells even when androgens are present
- Development of highly sensitive monoclonal antibodies that allow detection of native PCDH-PC protein on Western blots
- Preliminary immunostaining of human prostate cancer tissue microarrays indicate that PCDH-PC protein is selectively expressed in prostate cancer cells.
- Identification of a putative relationship between expression of PCDH-PC and SHH.

Reportable Outcomes

Published Manuscripts:

1. Yang, X., Chen, M.-W., Terry, S., Vacherot, F., Bemis, D.L., Capodice, J., Guo, Y. and Buttayan, R. (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. *Oncogene*, 25: 3436-3444.
2. Terry, S., Queires, L., Gil-Diez-de-Medina, S., Chen, M.-W., de la Taille, A., Allory, Y., Tran, P.-L., Abbou, C.C., Buttayan, R. and Vacherot, F. (2006) Protocadherin-PC promotes androgen-independent prostate cancer cell growth. *Prostate*, 66: 1100-1113.

Submitted Manuscripts:

1. Chen, M., Tanner, M., Levine, A., Levina, E., Ohouo, P. and Buttyan, R. (2008). Androgenic regulation of hedgehog signaling pathway components in prostate cancer cells. *Molecular Cancer*

Biological Resources:

1. shRNA expression vectors (2) that suppress PCDH-PC expression in prostate cancer cells.
2. Antisense Oligonucleotide (#181) that suppresses PCDH-PC expression in prostate cancer cells.
3. Monoclonal antibodies (3) that detect PCDH-PC protein on Western blots and in human prostate cancer cells in prostate cancer specimens.

Conclusions

Work in this project has so far led to the development of biological agents that specifically target and suppress PCDH-PC expression in prostate cancer cells and these agents are useful tools for identifying the role of PCDH-PC in the development of hormone-refractory prostate cancer and potentially for adjuvant therapeutics that might be used, in conjunction with hormone therapy, to improve treatment of advanced prostate cancer. Other work has led to the development of new monoclonal antibodies that recognize PCDH-PC protein and these antibodies might be useful to characterize PCDH-PC expression in human prostate tumor tissues.

References

1. Chen, M.-W., Vacherot, F., de la Taille, A., Shen, R., Gil-Diez-de-Medina, S., Chopin, D.K., Friedman, R. and Buttyan, R. (2002) Emergence of protocadherin T6 expression during the acquisition of apoptosis resistance by human prostate cancer cells. *Oncogene*, 21: 7861-7871.
2. Terry, S., Queires, L., Gil-Diez-de-Medina, S., Chen, M.-W., de la Taille, A., Allory, Y., Tran, P.-L., Abbou, C.C., Buttyan, R. and Vacherot, F. (2006) Protocadherin-PC promotes androgen-independent prostate cancer cell growth. *Prostate*, 66: 1100-1113.
3. Yang, X., Chen, M.-W., Terry, S., Vacherot, F., Chopin, D., Bemis, D., Kitajewski, J., Benson, M.C., Guo, Y. and Buttyan, R. (2005) A human- and male-specific protocadherin that acts through the wnt signaling pathway to induce neuroendocrine transdifferentiation of prostate cancer cells. *Cancer Res.* 65: 5263-5271.