Methylation-mediated Silencing of Genes Is Not Altered by Selenium Treatment of Prostate Cancer Cells

KAVITHA RAMACHANDRAN[2], LOIDA NAVARRO[2], EDNA GORDIAN[1], PARTHA MANAS DAS[2] and RAKESH SINGAL[1,2]

[1]Miami VA Medical Center, Miami, Florida 33125;
[2]Sylvester Cancer Center, University of Miami, Florida 33136, U.S.A.

Abstract. *Background: The role of selenium in reducing the risk of multiple cancers has been described in the literature. Although reports have described the antiproliferative and pro-apoptotic function of selenium by up-regulation of genes in these pathways, information is lacking on the target mechanisms of selenium on specific genes. This study examines whether selenium treatment alters the methylation status of epigenetically silenced genes in prostate cancer cells. Materials and Methods: Methylation of glutathione sulfotransferase pi (GSTP1) and Ras associated family 1A (RASSF1A) genes was studied using methylation sensitive PCR (MS-PCR). Gene expression was studied using Reverse Transcriptase PCR and Western Blotting. Results and Conclusion: Treatment of prostate cancer cells with selenium did not alter the expression of genes that were silenced by DNA methylation. Furthermore, the methylation status of these genes remained unaltered after treatment with seleno-DL-methionine.*

Prostate cancer is one of the leading malignancies in the United States and is second only to lung cancer in men. The mechanisms underlying the initiation, progression and metastatic dissemination of prostate cancer remains unclear. Several risk factors for prostate cancer have been identified, including age, race, dietary habits and androgen levels (1). Epidemiological studies have shown that diet plays a major role in the progression of prostate cancer (2). Changes in lifestyle have been identified as a major cause of occurrence of prostate cancer among migrating populations from countries of low incidence of the disease (3, 4). Since a large fraction of incidence of cancer has been attributed to diet alone, it has been hypothesized that lifestyle modifications could be one of the methods of cancer prevention. Hence, several substances that have the potential to inhibit cancer tumor formation were investigated that led to the development of a new area of research namely chemoprevention (5, 6).

Selenium is one of the many elements considered beneficial to our health; however it is a trace element, only needed in small amounts. Selenium is incorporated into proteins as selenocysteine (7). Dietary intake of selenium is of high significance since it helps to prevent cellular damage by oxygen radicals that can damage DNA, as well as other cellular components, and result in various diseases, including cancer. In addition to reports that found a potential benefit from selenium in cancer prevention, there is an ongoing study – Selenium and Vitamin E Cancer Prevention Trial (SELECT) – funded by the National Institutes of Health that will provide more information about the long term effects or benefits of this element specifically in prostate cancer prevention. The study looks at the role of selenium alone or in combination with vitamin E (8, 9). Selenium intake has been found to prevent various types of cancer including lung, colorectal, and prostate (5, 10). Other studies have shown that specific amounts of selenium enhance the immune defense in some types of cancer (11) and some viral infections (12).

Epigenetic silencing of tumor suppressor genes is a common event in most cancers, including prostate cancer. The major epigenetic alterations include DNA methylation and histone modifications. DNA methylation occurs by transfer of a methyl group by DNA methyl transferases (DNMTs) that target cytosine residues in 5'-CpG 3' dinucleotides. Inhibition of DNMTs results in reactivation of silenced genes, inhibitors thus emerge as effective drugs in the treatment of cancer (13, 14). The enzyme catalyses the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to C5 of cytosine within CpG residues after DNA replication and was found to be elevated in cancers including that of the colon (15). In addition, it was highly associated with promoter methylation resulting in gene silencing of tumor suppressor genes (16, 17). GSTP1 is the major gene that is responsible for defending the

Correspondence to: Rakesh Singal, M.D. 1550 NW 10th Avenue PAP 217 (M877), Miami, Florida 33136, U.S.A. Tel: +1 3052437678, Fax: +1 3052438561, e-mail: rsingal@med.miami.edu

Key Words: Chemoprevention, epigenetic silencing, DNA methylation, selenium.

prostate against oxidative DNA damage. However, in prostate cancer, this gene is a target of DNMTs and becomes hypermethylated in the early stages of the disease. Loss of genetic material from RASSF1A is one of the most frequent events in several types of human solid tumors. The CpG island promoter region of this gene is highly methylated in several human cancers, including that of the prostate. Investigators have reported that the DNA methyltransferase (DNMT1) inhibitor 5-azadeoxycytidine (azadC) protected rats that were fed a selenium deficient diet from the effects of carcinogens (18). However, there is lack of information on the mechanism of action of selenium compounds. Previous reports have shown that selenium compounds can inhibit the activity of methyl transferase (Mtase) activity (19). However, the effect of selenium treatment on methylation of epigenetically silenced genes has not been reported. In this study, we investigated whether treatment of prostate cancer cells with selenium compounds could reverse methylation and cause reactivation of epigenetically silenced tumor suppressor genes.

Materials and Methods

Cell culture. LNCaP, Du145, PC3 and HeLa cells (obtained from American Type Culture Collection) were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in a humidified incubator at 37˚C with 5% CO_2.

Drug treatment. Appropriate stock solutions of sodium selenite and seleno-DL-methionine were prepared in water and 0.0001N HCl respectively. Cells were seeded at a density of $2x10^6$ in 100 mm dishes for 24 h following which they were treated with 10 μM sodium selenite or seleno-DL-methionine in 0.0001 N HCl (150 μM) for 96 h. As controls, cells were treated with similar concentrations of the vehicle alone. As positive controls, cells were grown in 100 mm dishes and treated with 1 μm 5-aza-2'-deoxycytidine for 96 h.

RNA extraction and reverse transcriptase PCR (RT-PCR). RNA was extracted from the cell lines using RNA Stat 60 (Tel-Test Inc. Friendswood, TX, USA) as per the manufacturer's instructions. cDNA was prepared from 10 μg of RNA using the Reverse Transcription System from Promega (Madison, WI, USA) using random primers. Two microliters of the cDNA was used for the PCR reaction. A standard curve for IGFBP3 was generated using randomly primed cDNA prepared using RNA extracted from HeLa cells. The cDNA was used undiluted or diluted 10^{-1}, 10–2, 10^{-3}, 10^{-4} and 10^{-5}, and 2 μl of each dilution was amplified with primers designed for IGFBP3. The primer sets used for RT-PCR analysis were 5' GAT ACC CAG AAC TTC TCC TCC 3' (forward) and 5' CAT ACT TAT CCA CAC ACC AGC 3' (reverse). Conventional RT-PCR analysis was performed to study the expression of RASSF1A after selenium treatment. Primer sequences were 5' TCT GTG GCG ACT TCA TCT GG 3' (forward) and 5' GGA GGG TGG CTT CTT GCT G 3' (reverse).

DNA extraction, bisulfite treatment and methylation-specific PCR (MS-PCR). DNA from drug-treated cells was extracted using DNA Stat 60 (Tel-Test Inc.). Genomic DNA was treated with sodium bisulfite under conditions that convert unmethylated cytosine to uracil while the 5-methylcytosine remains unchanged. The bisulfite conversion reaction was carried out by incubating 5 μg DNA with a 5 M bisulfite solution and 100 mM hydroquinone, pH 5.0 at 50˚C for 4 hours. This was followed by desulfonation *via* the addition of 3 M NaOH, and desalting using a QIAquick column (Qiagen, CA, USA). MS-PCR analysis for GSTP1 and RASSF1A was performed using methylated and unmethylated primers as described elsewhere (20, 21).

Western Blotting. Whole cell lysates were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad CA, USA). Protein concentrations of the lysates were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA). For Western blot analysis, the proteins were resolved on a 15% SDS-PAGE gel and blotted onto a nitrocellulose membrane. The blot was then probed with rabbit anti-human GSTP1 antibody (1:2000 dilution; Assay Designs, Ann Arbor, MI, USA) and detected using anti-rabbit Ig-HRP (GE Healthcare, Piscataway, NJ, USA), then visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce).

Results

Selenium treatment up-regulates IGFBP3 in LNCaP prostate cancer cells. Earlier reports described anti-proliferative effects of selenium treatment in androgen-dependent LNCaP cells with no effect on androgen-insensitive PC3 cells (2). Treatment with selenium was shown to up-regulate the expression of insulin-like growth factor binding protein 3 (IGFBP3) and retinoic X receptor alpha (RXRa) in PC3 cells and prostate cancer tissues (22). In order to optimize the treatment conditions, we treated LNCaP cells with seleno-DL-methionine and sodium selenite. IGFBP3 expression after 24, 48 and 96 h was measured using quantitative RT-PCR analysis. We observed similar effects with both selenium compounds: after 96 h there was an approximately 2.7-fold increase in IGFBP3 expression relative to treatment with the vehicle (Figure 1). These results are consistent with the previously reported effects of selenium treatment on gene expression in prostate cancer cells (22).

Selenium treatment does not reactivate GSTP1 expression in LNCaP prostate cancer cells. Previous studies have shown that treatment of androgen-responsive LNCaP cells with selenium (such as seleno-DL-methionine) resulted in inhibition of cell proliferation in a time- and dose-dependent manner (2). The mechanism of action of selenium compounds on gene regulation has not yet been elucidated. Selenium was also shown to inhibit Mtase activity and growth in human colon carcinoma HCT116 cells (19). However, the effect of selenium on methylation of genes has not yet been described. Using GSTP1 as a marker, we examined whether seleno-DL-methionine treatment results in demethylation and re-expression of genes in LNCaP cells. As control, cells were treated with the known demethylating

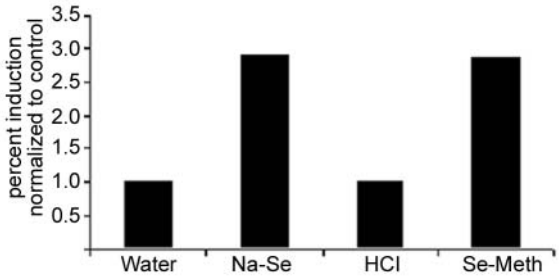

Figure 1. *IGFBP3 expression in LNCaP cells treated with selenium compounds. Cells were treated with sodium selenite in water (10 μM) or seleno-DL-methionine in 0.0001 N HCl (150 μM) for 96 h. As controls, the cells were treated with the respective vehicles alone. RNA was then extracted, reverse transcribed and the percentage of expression was plotted after quantitative RT-PCR analysis.*

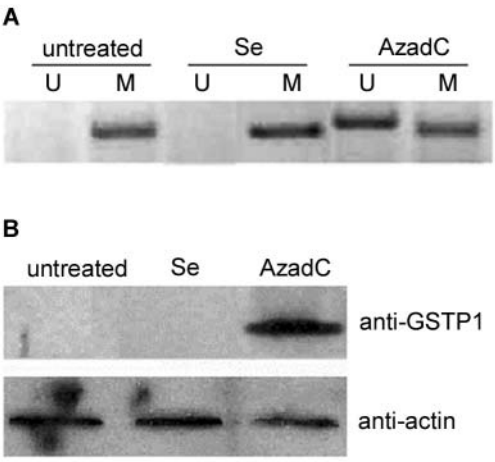

Figure 2. *A) GSTP1 MS-PCR analysis after selenium treatment. LNCaP cells were treated with 150 μM seleno-DL-methionine (Se) or 1 μM azadeoxycytidine (AzadC) for 96 h. After treatment, DNA was extracted, treated with bisulfite and analyzed using methylation specific polymerase chain reaction (MS-PCR) with methylation specific primers. U- unmethylated, M- methylated. B) Western Blot Analysis. LNCaP cells were treated with 150 μM of seleno-DL-methionine (Se) or 1 μM azadeoxycytidine (AzadC). After 96 h, cells were harvested and whole cell lysates were prepared. The lysates were resolved using SDS-PAGE and probed with anti-GSTP1 antibody. Top panel shows levels of GSTP1 in untreated, selenium treated and AzadC-treated cells. The blots were stripped and reprobed with anti-actin antibody to ensure equal loading (bottom panel).*

agent, 5-aza-2'-deoxycytidine. The methylation status of the GSTP1 gene promoter after drug treatment was analyzed using MS-PCR. Treatment with 1 μM 5-aza-2'-deoxycytidine for 96 h resulted in significant demethylation of the promoter. However, the methylation status of the gene remained unaltered after treatment with 150 μM of seleno-DL-methionine (Figure 2A). Western blotting analysis showed an increase in the levels of GSTP1 protein upon treatment with 1 μM 5-aza-2'-deoxycytidine, whereas no protein expression was seen after treatment with selenium compounds (Figure 2B).

Effect of selenium treatment in androgen insensitive prostate cancer cells. Using gene expression profiling, Schlicht *et al.*, have identified up-regulation of IGFBP3 and RXRα in PC3 cells (22). Treatment with selenium was shown to cause G_1 arrest in androgen-dependent LNCaP cells concomitant with the up-regulation of cyclin-dependent kinase inhibitors while there was no effect in androgen-negative PC3 cells (2). We examined the effect of selenium on RASSF1A in Du145 and PC3 cells. RASSF1A is epigenetically deregulated by DNA methylation in both the cell lines. After treatment with 150 μM seleno-DL-methionine for 96 h, no change in expression of RASSF1A was observed using RT-PCR analysis. MS-PCR analysis showed that the gene remained methylated after the drug treatment (Figure 3).

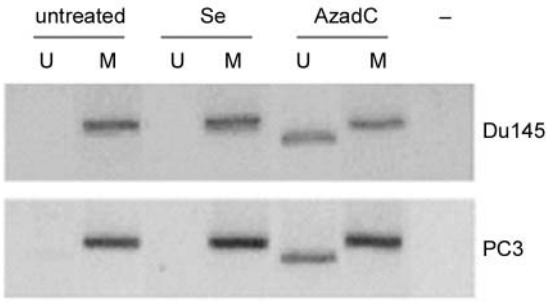

Figure 3. *RASSF1A MS-PCR analysis after treatment with selenium compounds. Du145 and PC3 prostate cancer cell lines were treated with 150 μM seleno-DL-methionine (Se) or 1 μM azadeoxycytidine (AzadC). After 96 h of treatment, DNA was extracted, treated with bisulfite and analyzed through methylation specific polymerase chain reaction with specific primers. U, unmethylated, M, methylated, –, negative control.*

Discussion

Selenium, an essential nutrient found in trace amounts in food, has been demonstrated to have a chemopreventive role against various cancers (11, 23). Several studies have demonstrated an inverse relationship between selenium intake and prostate cancer risk (8, 9, 24, 25). Nutritional Prevention of Cancer was a pioneering study by Clark *et al.*, to test the chemopreventive action of selenium against skin cancer (10). Although selenium treatment did not protect against cancer of the skin, results showed that selenium supplementation reduced the incidence of other carcinomas, including lung, colorectal and prostate. This observation formed the basis for subsequent investigations on effects of selenium on cancer including the ongoing SELECT study (26). Anti-proliferative effects of selenium were reported in androgen-dependent LNCaP and

androgen-insensitive PC3 and Du145 prostate cancer cell lines (2, 24, 27-30). Selenium was shown to inhibit growth, block cell cycle progression and induce apoptosis in prostate cancer cells (27). Selenium treatment resulted in increased androgen receptor (AR) degradation and reduced nuclear AR, thus disrupting AR signaling (27). Selenium was also shown to act synergistically with vitamin E to inhibit growth of LNCaP cells *via* two distinct pathways (31). In addition, *in vivo* studies using mouse xenograft models have demonstrated anti-tumorigenic properties of selenium (32).

Although selenium treatment was found to result in up-regulation of genes responsible for blocking cell cycle progression and subsequent growth arrest, the mechanism of gene induction has yet to be described. Whether selenium can reactivate genes by reversing epigenetic silencing has not been addressed in literature yet. Hence, we carried out this study to examine the re-expression of genes commonly inactivated by DNA methylation in prostate cancer. GSTP1 methylation is an early event in prostate cancer and was demonstrated in high-grade prostatic intraepithelial neoplasia (PIN) lesions and prostate cancer, while it was absent in normal prostate tissues (20, 33). This identified GSTP1 methylation as the most potential biomarker for early detection of the disease (34). We examined the effect of selenium treatment on the expression of GSTP1 in LNCaP, in which the promoter is methylated leading to repression of gene expression (20). While selenium treatment was effective in up-regulating IGFBP3, as has been previously described, it did not induce expression of GSTP1 in LNCaP prostate cancer cells. Loss of genetic material from RASSF1A at 3p21.3 is one of the most frequent events in several types of human solid tumors. The CpG island promoter region of this gene is highly methylated in several human cancers, including that of the prostate (35). Complete silencing and methylation of RASSF1A promoter was observed in widely used prostate carcinoma cell lines including LNCaP, PC3 and Du145. Our results on selenium treatment of LNCaP, Du145 and PC3 cells showed no effect on RASSF1A methylation on expression in these cells.

Conclusion

Our results show that selenium does not alter the epigenetic state of the studied genes. Further, selenium treatment does not reactivate genes that are predominantly regulated by DNA methylation.

Acknowledgements

This study was supported by the Department of Veterans Affairs and a grant from the United States Department of Defense (DOD) to RS.

References

1 Ross RK and Henderson BE: Do diet and androgens alter prostate cancer risk *via* a common etiologic pathway? J Natl Cancer Inst *86*: 252-254, 1994.

2 Venkateswaran V, Klotz LH and Fleshner NE: Selenium modulation of cell proliferation and cell cycle biomarkers in human prostate carcinoma cell lines. Cancer Res *62*: 2540-2545, 2002.

3 Haenszel W and Kurihara M: Studies of Japanese migrants. I. Mortality from cancer and other diseases among Japanese in the United States. J Natl Cancer Inst *40*: 43-68, 1968.

4 Staszewski J and Haenszel W: Cancer mortality among the Polish-born in the United States. J Natl Cancer Inst *35*: 291-297, 1965.

5 Sonn GA, Aronson W and Litwin MS: Impact of diet on prostate cancer: a review. Prostate Cancer Prostatic Dis *8*: 304-310, 2005.

6 Shukla S and Gupta S: Dietary agents in the chemoprevention of prostate cancer. Nutr Cancer *53*: 18-32, 2005.

7 Rayman MP: Selenium in cancer prevention: a review of the evidence and mechanism of action. Proc Nutr Soc *64*: 527-542, 2005.

8 Neill MG and Fleshner NE: An update on chemoprevention strategies in prostate cancer for 2006. Curr Opin Urol *16*: 132-137, 2006.

9 Lowe JF and Frazee LA: Update on prostate cancer chemoprevention. Pharmacotherapy *26*: 353-359, 2006.

10 Clark LC, Combs GF Jr, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL Jr, Park HK, Sanders BB Jr, Smith CL and Taylor JR: Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. Jama *276*: 1957-1963, 1996.

11 Kiremidjian-Schumacher L, Roy M, Glickman R, Schneider K, Rothstein S, Cooper J, Hochster H, Kim M and Newman R: Selenium and immunocompetence in patients with head and neck cancer. Biol Trace Elem Res *73*: 97-111, 2000.

12 Broome CS, McArdle F, Kyle JA, Andrews F, Lowe NM, Hart CA, Arthur JR and Jackson MJ: An increase in selenium intake improves immune function and poliovirus handling in adults with marginal selenium status. Am J Clin Nutr *80*: 154-162, 2004.

13 Das PM and Singal R: DNA methylation and cancer. J Clin Oncol *22*: 4632-4642, 2004.

14 Laird PW: Cancer epigenetics. Hum Mol Genet 14 Spec No *1*: R65-76, 2005.

15 el-Deiry WS, Nelkin BD, Celano P, Yen RW, Falco JP, Hamilton SR and Baylin SB: High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. Proc Natl Acad Sci USA *88*: 3470-3474, 1991.

16 Gopisetty G, Ramachandran K and Singal R: DNA methylation and apoptosis. Mol Immunol *43*: 1729-1740, 2006.

17 Baylin SB: DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol *2(Suppl 1)*: S4-11, 2005.

18 Davis CD and Uthus EO: Dietary selenite and azadeoxycytidine treatments affect dimethylhydrazine-induced aberrant crypt formation in rat colon and DNA methylation in HT-29 cells. J Nutr *132*: 292-297, 2002.

19 Fiala ES, Staretz ME, Pandya GA, El-Bayoumy K and Hamilton SR: Inhibition of DNA cytosine methyltransferase by chemopreventive selenium compounds, determined by an improved assay for DNA cytosine methyltransferase and DNA cytosine methylation. Carcinogenesis *19*: 597-604, 1998.
20 Singal R, van Wert J and Bashambu M: Cytosine methylation represses glutathione S-transferase P1 (GSTP1) gene expression in human prostate cancer cells. Cancer Res *61*: 4820-4826, 2001.
21 Tomizawa Y, Kohno T, Kondo H, Otsuka A, Nishioka M, Niki T, Yamada T, Maeshima A, Yoshimura K, Saito R, Minna JD and Yokota J: Clinicopathological significance of epigenetic inactivation of RASSF1A at 3p21.3 in stage I lung adenocarcinoma. Clin Cancer Res *8*: 2362-2368, 2002.
22 Schlicht M, Matysiak B, Brodzeller T, Wen X, Liu H, Zhou G, Dhir R, Hessner MJ, Tonellato P, Suckow M, Pollard M and Datta MW: Cross-species global and subset gene expression profiling identifies genes involved in prostate cancer response to selenium. BMC Genomics *5*: 58, 2004.
23 Combs GF Jr, Clark LC and Turnbull BW: Reduction of cancer risk with an oral supplement of selenium. Biomed Environ Sci *10*: 227-234, 1997.
24 Dong Y, Zhang H, Hawthorn L, Ganther HE and Ip C: Delineation of the molecular basis for selenium-induced growth arrest in human prostate cancer cells by oligonucleotide array. Cancer Res *63*: 52-59, 2003.
25 Combs GF Jr: Status of selenium in prostate cancer prevention. Br J Cancer *91*: 195-199, 2004.
26 Cook ED, Moody-Thomas S, Anderson KB, Campbell R, Hamilton SJ, Harrington JM, Lippman SM, Minasian LM, Paskett ED, Craine S, Arnold KB and Probstfield JL: Minority recruitment to the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Clin Trials *2*: 436-442, 2005.
27 Chun JY, Nadiminty N, Lee SO, Onate SA, Lou W and Gao AC: Mechanisms of selenium down-regulation of androgen receptor signaling in prostate cancer. Mol Cancer Ther *5*: 913-918, 2006.
28 Dong Y, Lee SO, Zhang H, Marshall J, Gao AC and Ip C: Prostate specific antigen expression is down-regulated by selenium through disruption of androgen receptor signaling. Cancer Res *64*: 19-22, 2004.
29 Zhao H, Whitfield ML, Xu T, Botstein D and Brooks JD: Diverse effects of methylseleninic acid on the transcriptional program of human prostate cancer cells. Mol Biol Cell *15*: 506-519, 2004.
30 Jiang C, Ganther H and Lu J: Monomethyl selenium-specific inhibition of MMP-2 and VEGF expression: implications for angiogenic switch regulation. Mol Carcinog *29*: 236-250, 2000.
31 Venkateswaran V, Fleshner NE and Klotz LH: Synergistic effect of vitamin E and selenium in human prostate cancer cell lines. Prostate Cancer Prostatic Dis *7*: 54-56, 2004.
32 Corcoran NM, Najdovska M and Costello AJ: Inorganic selenium retards progression of experimental hormone refractory prostate cancer. J Urol *171*: 907-910, 2004.
33 Lin X, Asgari K, Putzi MJ, Gage WR, Yu X, Cornblatt BS, Kumar A, Piantadosi S, DeWeese TL, De Marzo AM and Nelson WG: Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. Cancer Res *61*: 8611-8616, 2001.
34 Bastian PJ, Nakayama M, De Marzo AM and Nelson WG: GSTP1 CpG island hypermethylation as a molecular marker of prostate cancer. Urologe A *43*: 573-579, 2004.
35 Jeronimo C, Henrique R, Hoque MO, Mambo E, Ribeiro FR, Varzim G, Oliveira J, Teixeira MR, Lopes C and Sidransky D: A quantitative promoter methylation profile of prostate cancer. Clin Cancer Res *10*: 8472-8478, 2004.

Received September 22, 2006
Revised December 7, 2006
Accepted December 13, 2006