Prostate Cancer and Prostatic Diseases (2004) 7, 327–332
 1365-7852/04 $30.00
www.nature.com/pcan

Type IV collagenase (matrix metalloproteinase-2 and -9) in prostate cancer

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Background: The type IV collagenases/gelatinases matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) play an important role in cancer invasion and metastasis. In the present study, we measured the expression of mRNAs and enzymatic activities of MMP-9 and -2 in prostate tissues and serum samples from men with or without prostate cancer.

Methods: A total of 44 tissue samples (three from healthy volunteers, 21 from patients with benign prostate hyperplasia, 10 from patients with localized prostate cancer and 10 from patients with metastatic disease) and 71 serum samples were collected (20 from healthy volunteers, 26 from patients with benign prostatic hyperplasia, 10 from patients with localized cancer, 15 from patients with metastatic cancer). The level of mRNA for MMP-2 and -9 was determined by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The enzymatic activity of MMPs was determined by zymography.

Results: Expression of MMP-9 mRNA was significantly higher in malignant than in nonmalignant prostate tissues ($P<0.001$), while no significant difference of MMP-2 expression was detected in different prostate tissues. Results of zymography showed that there was significant difference in the enzymatic activity of MMP-9, but not MMP-2, among normal prostate, BPH, localized and metastatic prostate cancer tissues, serum samples ($P<0.05$). The active form of MMP-2, with a molecular mass of 62 kDa, was detected in normal prostate, BPH and prostate cancer tissues, but not in the serum samples. Moreover, there was a significant difference in the ratio of the active form (62 kDa) and proform (72 kDa) of MMP-2 among normal, BPH and prostate cancer tissues. This ratio was further increased in metastatic prostate cancer tissues.

Conclusion: The activity of MMP-9 and the ratio of active form/proform of MMP-2 are associated with the progression and metastasis of prostate cancer.

Prostate Cancer and Prostatic Diseases (2004) **7,** 327–332. doi:10.1038/sj.pcan.4500750
Published online 7 September 2004

Keywords: metastasis; MMP-2; MMP-9; zymography; RT-PCR

Introduction

Tumor metastasis involves extensive interactions between the invading cancer cells and the surrounding stromal cells. Such interactions promote degradation of the extracellular matrix (ECM) by specialized proteolytic enzymes, which are produced by both the cancer cells and the stromal cells, and are likely to affect both primary and metastatic sites. Among these enzymes, urokinase and a variety of matrix matalloprotienases (MMPs) play important roles.[1–4]

Among different MMPs, MMP-2 (or gelatinase A) and MMP-9 (or gelatinase B) collectively referred to as type IV collagenases or gelatinases, have been found to be specifically associated with prostate cancer metastasis. Elevated levels of MMP-2 and -9 in the plasma and urine have been correlated with metastasis in prostate cancer patients.[5,6] Secretion of MMP-2 and -9 induce tumor

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Received 3 March 2004; revised 29 April 2004; accepted 14 June 2004; published online 7 September 2004

angiogenesis in prostate cancer cells.[7,8] Pettaway and colleagues found that the expression of MMP-2 and -9 mRNA, in comparison with E-cadherin expression, at biopsy could predict advanced prostate cancer at radical prostatectomy.[9,10]

In the present study, we investigated the expression of MMP-2 and -9 in prostate tissue samples using a semiquantitative RT-PCR. Zymographic techniques are used for the detection of enzymatic activities.[11] We found that MMP-9 was associated with prostate cancer in tissue and serum specimens. We also detected a change in the ratio of the active form and proform of MMP-2 in prostate cancer.

Materials and methods

Tissue and serum samples

BPH and prostate cancer tissue samples were obtained during open prostatectomy surgery, and immediately frozen in liquid nitrogen and stored at −80°C. Prostatic tissues were obtained from automobile accident victims (age 22–28 y, median 24), patients with BPH (age 54–67 y, median 63), patients with organ-confined (age 57–72 y, median 69), and patients with metastasis disease (age 58–74 y, median 71). For gelatin zymography, three normal prostate, 21 BPH, 10 organ-confined prostate cancer and 10 metastatic tissues were sampled.

Serum samples were collected from 10 patients with organ-confined prostate cancer (age 57–76 y, median 71), 15 patients with metastatic prostate cancer (age 58–74 y, median 71), 26 patients with BPH (age 51–69 y, median 66) and 20 healthy volunteers (10 male and 10 female; age 23–45 y, medium 27).

Procurement of the above tissue and serum specimens has been approved by the Institutional Review Board of Nankai University. Clinical specimens received the exemption status, while specimens from automobile accident victims received consent for research from family members.

RNA extraction

Total RNA was prepared from tissue samples using Trizol (Gibco) according to the manufacturer's instructions. RNA was suspended in DEPC-H_2O and stored at −80°C until use. The purity of the RNA was established by reading the optical density of each sample at 260 and 280 nm, using Ultrospec 1100 pro Spectophotometer (American Pharmacia).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

An aliquot of 1.0 μg of RNA was added to RNase-free water to a final volume of 10 μl, denatured for 5 min at 72°C and cooled immediately on ice, followed by the addition of RT mixture (10 μl), which contained first-strand buffer, 200 U of Moloney murine leukemia virus, 20 U of RNasin, 10 mM DTT, 4.75 μM random hexamers and 500 mM deoxynucleotides (Promega, Madison, WI, USA). The reaction was carried out at 37°C for 2 h, followed by an enzyme inactivation step for 5 min at 95°C. The resulting cDNA was stored at −20°C until use.

The PCR reaction was carried out in 25 μl of final volume containing 1.0 μl of cDNA, 0.5 μM of each primer, 1 × PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.75 U Ampli*Taq* Gold DNA polymerase. The following primers were used:

MMP-2 forward primer, 5-ACC TGG ATG CCG TCG TGG AC-3;

MMP-2 reverse primer, 5-TGT GGC AGC ACC AGG GCA GC-3 (for amplification of a 447-bp product for human MMP-2, accession number J03210);

MMP-9 forward primer, 5-GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC-3;

MMP-9 reverse primer, 5-GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT-3 (for amplification of a 640-bp product for human MMP-9, accession number NM-004994);

β-microglobulin forward primer, 5-ATG CCT GCC GTG TGA ACC ATG T-3;

β-microglobulin reverse primer, 5-AGA GCT ACC TGT GGA GCA ACC T-3 (for amplification of a 285-bp product for human β-microglobulin, accession number NM-004048).

The PCR reaction was conducted with the following steps: after an initial denaturation step of 5 min at 94°C, 34 cycles of denaturation at 95°C for 25 s, primer annealing at 58°C for 1 min and extension at 72°C for 1 min were performed (21 cycles for β-microglobulin). A final extension step was performed at 72°C for 5 min in order to complete the PCR reaction. Possible DNA contamination was monitored by performing PCR in the same conditions without the addition of cDNA. PCR for the housekeeping gene (β-microglobulin) and MMP genes were performed at the same annealing temperature in the same cycle run for all samples. This procedure was followed so that comparison of gene expression in different samples was performed under the same conditions of amplification.

Semiquantitative analysis of PCR products

A detailed procedure for the semiquantitative analysis of PCR products for MMP-2 and -9 was reported earlier.[12] In the present study, we used β-microglobulin as the internal standard. The PCR products for MMPs and β-microglobulin were analyzed by 1.5% agarose gel electrophoresis. PCR products were visualized by ethidium bromide staining. The density of each band was measured by a computer-assisted image analysis system (Syngene). Integrated density intensity of the band for β-microglobulin, the housekeeping gene, of each sample was arbitrarily set as 1 and the density of the band of individual genes was adjusted to this value.

Zymography

The zymography was conducted according to established reports[11,13] with minor modifications. Briefly, fresh prostatic issues were cut into small pieces and mixed with ice-cold extraction buffer (50 mM Tris-HCl pH7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA). The mixture was homogenized at 4°C and centrifuged at 10 000 × g for 10 min. The supernatant fraction from each

preparation was transferred into a new tube, and the protein concentration was estimated, using the Bio-Rad protein assay reagent (Bio-Rad). An aliquot of 40 μg of protein was mixed with the sample buffer in nonreducing conditions (in the absence of mercaptoethanol or DTT) and loaded onto a 10% polyacrylamide gel, which has been incorporated with 1.0 mg/ml gelatin (Sigma) for electrophoresis. The serum samples were treated with the same protocol. Conditions for electrophoresis were 100 V for 1.5 h alongside with a broad-range molecular weight marker (Bio-Rad). At the conclusion of electrophoresis, SDS was removed by washing the gel twice for 30 min with 2.5% Triton X-100 in 50 mM Tris-HCl (pH 7.5) and once for 20 min with 50 mM Tris-HCl (pH 7.5). The gels were incubated overnight at 37°C with 50 mM of Tris-HCl (pH 7.5), 0.15 M of NaCl, 10 mM of $CaCl_2$, 0.1% of Triton X-100. Staining was carried out for 1 h at room temperature with 0.5% Coomassie brilliant R-250 in 45% methanol and 10% acetate, followed by destaining with 45% methanol and 10% acetate until clear bands over a blue background were observed. Purified human MMP-2 and -9 (Chemicon) were loaded at 10 ng per lane as controls. The relative intensity of each gel was normalized against the respective controls and was expressed as the fraction of the control. The intensity of bands corresponding to MMP-2 and -9 was measured using a computer-assisted image analysis system (Syngene). The ratio of the bands of the active form (62 kDa) over the proform (72 kDa) of MMP-2 (active form/proform) was also calculated.

Statistics analysis

The normalized expression of MMP-2 or -9 was estimated by their median values and ranges. Comparison of the means of different groups was performed using *t*-tests. A *P*-value less than 0.05 was considered as statistically significant. The Statistical Package for Social Science (SPSS) was used for the present study.

Results

RT-PCR analysis of MMP-2 and -9 expression in prostate tissue samples

Figure 1 shows the result of semiquantitative RT-PCR analysis of MMP-2 and -9 expression in human prostate tissues. A similar procedure was described earlier.[12] The density of the band of individual genes was normalized with that of the housekeeping gene (β-microglobulin). There was no significant difference in the normalized expression of MMP-2 in normal, BPH and prostate cancer tissues. However, for MMP-9, the median normalized expression was significantly different between nonmalignant and malignant prostate tissues ($P<0.001$), and there was a further significant increase in the metastatic prostate cancer compared to that of the localized prostate cancer.

Zymography analysis of MMP-2 and -9 expression in prostate tissue samples

Enzymatic activities for MMP-2 and -9 were determined by the gelatinolytic activity in SDS-PAGE zymograms. Figure 2 shows an example of zymography for MMP-2 and -9 in prostate tissue samples. MMP-2 showed two distinct bands. The proform has a higher molecular weight and the active form has a lower molecular weight. As indicated in Figure 3a, there was a significant difference in the activity of MMP-9 between normal

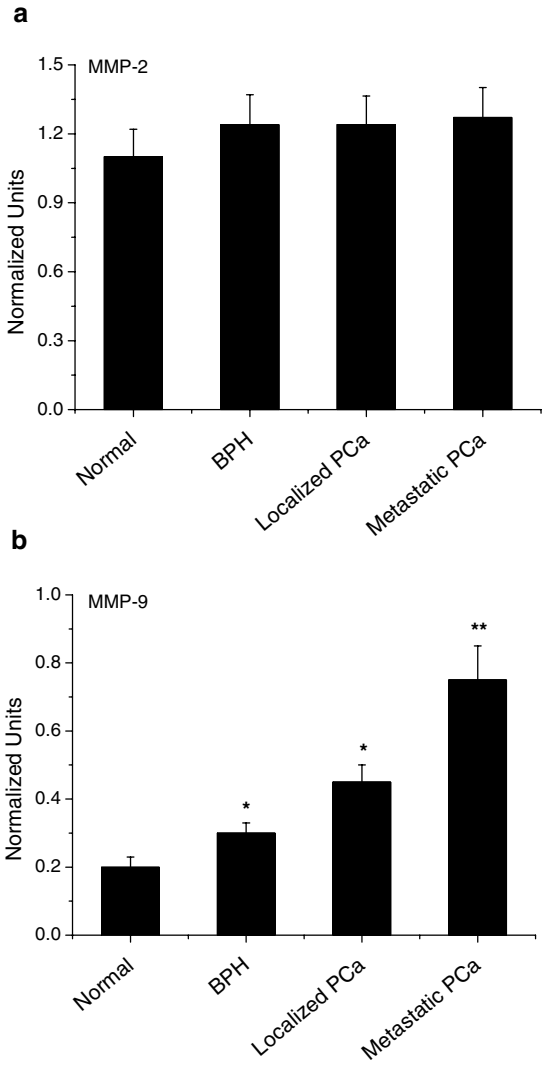
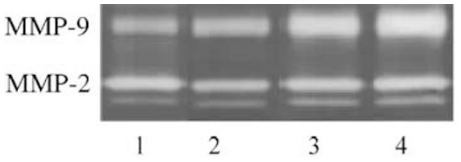


Figure 1 *Normalized expression of MMP-2 and -9 in normal, BPH, localized and metastatic prostate cancer tissues in comparison to the housekeeping gene* β*-microglobulin. Vertical bars denote standard deviation. *Denotes that the value is significantly different from that of the normal ($P<0.05$). **Denotes that the value is significantly different from that of the BPH ($P<0.05$).*



Lane1, normal prostate; Lane 2, BPH; Lane 3, localized prostate cancer; Lane 4, metastatic prostate cancer.

Figure 2 *Analysis of enzymatic activity of MMP-9 and -2 in prostate tissue samples by zymography.*

tissues and BPH tissues ($P<0.05$), as well as between BPH and metastatic prostate cancer ($P<0.05$). The enzymatic activity for MMP-9 in metastatic prostate cancer was about three-fold higher than that in BPH and about nine-fold higher than that in normal tissues.

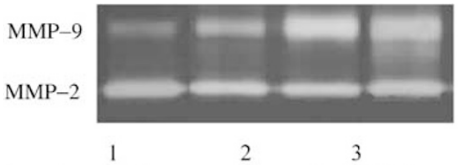
Figure 3b shows that, although the enzymatic activity for MMP-2 in the normal prostate was significantly lower than that BPH and prostate cancer tissues ($P<0.001$), the MMP-2 activity was not significantly different between BPH and prostate cancer ($P>0.05$). Interestingly, there was a significant difference ($P<0.01$) in the ratio of the active form of MMP-2 (62 kDa) over the proform (72 kDa) between the normal prostate and BPH, as well as between BPH, and metastatic prostate cancer tissues (Figure 3c).

Zymography analysis of MMP-2 and -9 expression in prostate serum samples

Figure 4 shows an example of the zymography for MMP-2 and -9 in serum samples. Figure 5a shows that, similar to the values observed in prostate tissues, the enzymatic activity for MMP-9 was significantly different between the normal prostate and BPH tissues ($P<0.05$), as well as between BPH and metastatic prostate cancer ($P<0.05$). Also similar to the findings in the tissues, there was no significant difference in the enzymatic activity for MMP-2 among the normal prostate, BPH and prostate cancer tissues. The active form of MMP-2 was not detected in any of the serum samples.

Discussion

Results of the present study demonstrated that the expression of MMP-9 mRNA as well as the enzymatic activity was significantly higher in malignant than in nonmalignant prostate tissues. The present study also indicated that, although the expression of MMP-2 mRNA was not significantly different. However, the ratio of the active form of MMP-2 (62 kDa) over its proform (72 kDa) was significantly different between the normal prostate and BPH, as well as between BPH and metastatic prostate cancer tissues. Findings from the serum specimens also



Lane1, normal prostate; Lane 2, BPH; Lane 3, localized prostate cancer; Lane 4, metastatic prostate cancer.

Figure 4 *Zymography analysis of activity of MMP-9 and -2 in serum samples.*

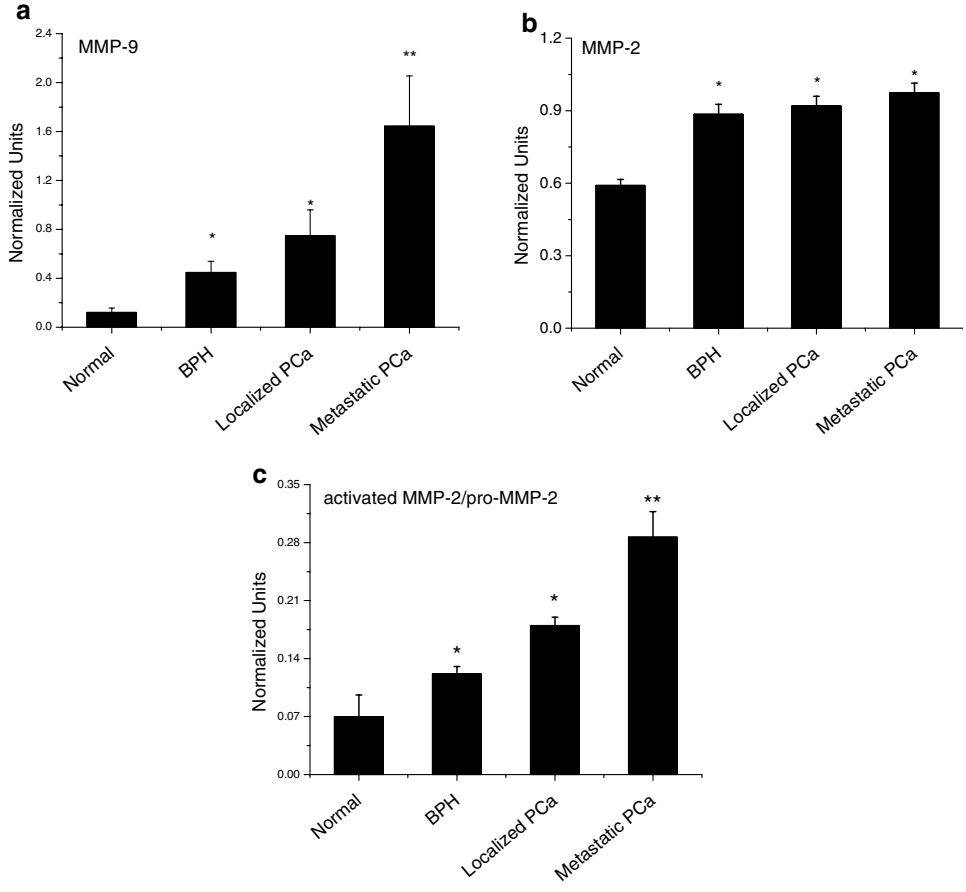


Figure 3 *Relative activity of MMP-2 and -9 in prostate tissue samples. Vertical bars denote standard deviation. *Denotes that the value is significantly different from that of the normal ($P<0.05$). **Denotes that the value is significantly different from that of the BPH ($P<0.05$).*

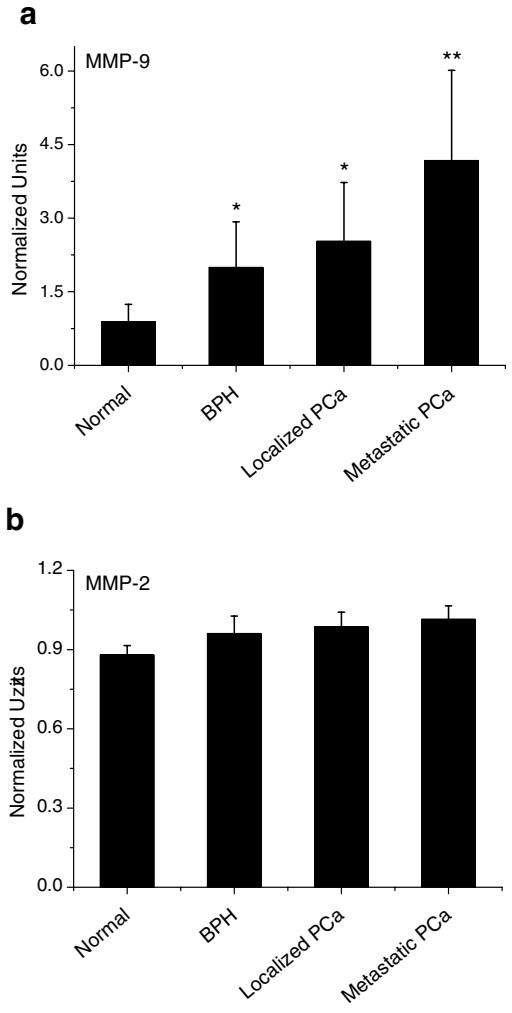


Figure 5 *Relative activity of MMP-2 and -9 in serum samples. Vertical bars denote standard deviation. *Denotes that the value is significantly different from that of the normal ($P<0.05$). **Denotes that the value is significantly different from that of the BPH ($P<0.05$).*

demonstrated a significant difference in MMP-9, but not in MMP-2, among the normal subjects, BPH and prostate cancer patients. Our study also demonstrated that the active form of MMP-2 was not found in any of the serum specimens. These findings offer a possibility that MMP-9 in tissues and in sera and the ratio of active/proform for MMP-2 in prostate tissues could be potential prognostic markers for prostate cancer patients.

Many studies have demonstrated that MMPs correlate with cancer metastases.[5–10] Although MMP-2 and -9 were detected in tissues and cells of the prostate,[9] MMP-2 was also expressed in the stromal cells.[12] MMP-2 and -9 were thought to be the key matrix matalloproteinases involved in cancer cell invasion and metastases, since their overexpression could be induced by many factors such as cytokines, growth factors and oncogenes.[13–17] In the present study, we further demonstrated that MMP-9 levels in prostate tissues could also be an important MMP in cancer progression and metastasis. The activity of MMP-9 in metastatic prostate cancer tissues was about three-fold higher than that in BPH and about nine-fold higher than that in normal tissues. Our future studies will be directed toward factors in prostate cancer that regulate expression of MMPs.

Although there was no significant difference between BPH and prostate cancer samples in enzymatic activity of MMP-2 and in the level of MMP-2 mRNA. The activated form of MMP-2 was only detected in tissue samples, not in any serum samples. The ratio of activated MMP-2/proform in metastatic prostate cancer samples was about two-fold higher than that in BPH samples. These results suggest that the activity of MMP-2 alone could not differentiate BPH from prostate cancer. These observations seem to underscore the potential role of MMP-2 in prostate cancer metastasis. However, the significant difference in the ratio of activated MMP-2/proform between BPH samples and prostate cancer samples suggest that BPH and prostate cancer may have such a functional difference.

Brown *et al*[18,19] showed for the first time by the approach of gelatin zymography that the ratio of active-/proform of MMP-2 correlated with lymph node metastasis in the human breast and lung cancers. Subsequent studies demonstrated that this finding could apply to many human cancers including thyroid cancer, oral squamous cell carcinoma, stomach carcinomas, breast carcinomas and non-small cell carcinoma of the lung.[20] Results of the present study also confirmed the above conclusion for prostate cancer.

Serum levels of MMPs were found to be correlated with invasion and metastasis of many malignancies, including human lung cancers as well as breast[21] and gastrointestinal cancers.[22] Results of the present study verified that MMP-9 levels in the serum specimens correlated with the presence of malignancy, as well as with the metastatic status of prostate cancer. In serum samples, the activity of MMP-9 in patients with metastatic prostate cancer was about two-fold higher than that in patients with BPH.

In the present study, we were unable to detect any correlation in changes in serum MMP-2 levels between BPH patients and patients with prostate cancer. However, Gohji *et al*[6] detected MMP-2 in serum specimens using a monoclonal antibody and found that the density of MMP-2 in serum was associated with the development and extension of prostate cancer and that the serum MMP-2 level indicated the degree of prostate cancer extension. The discrepancy between this study and the study by Gohji's group is unclear. It is likely that a difference in detection methodology used in two studies could account for such a difference.

In conclusion, our results indicated that the expression of MMP-9 in prostate tissues and serum and the ratio of activated MMP-2/proform in tissues were associated with metastatic prostate carcinoma. These findings were supported by a most recent independent report.[23] These parameters may be useful as a prognostic marker for human prostate cancer.

Acknowledgements

This research was funded by the following grants: 3×3 Canada China Biotechnology Seed Grant, the National Natural Science Foundation of China (Grant No. 30271297), and the United States Department of Health

and Human Services, National Institutes of Health (NCI) Prostate Cancer SPORE (Grant No. P50 CA90386-01).

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