vectors encoding FLAG-tagged TRIM68 and HA-tagged TIP60 were transfected into HEK293T cells. The cell lysates were subjected to immunoprecipitation with anti-HA antibody, and then immunoblot analysis was performed using anti-FLAG antibody. An *in vivo* binding assay showed that TRIM68 specifically interacts with TIP60 (Fig. 4*A*). To further determine whether TRIM68 functionally interacts with TIP60, we performed an AR transactivation assay. LNCaP cells were cotransfected with expression vectors encoding

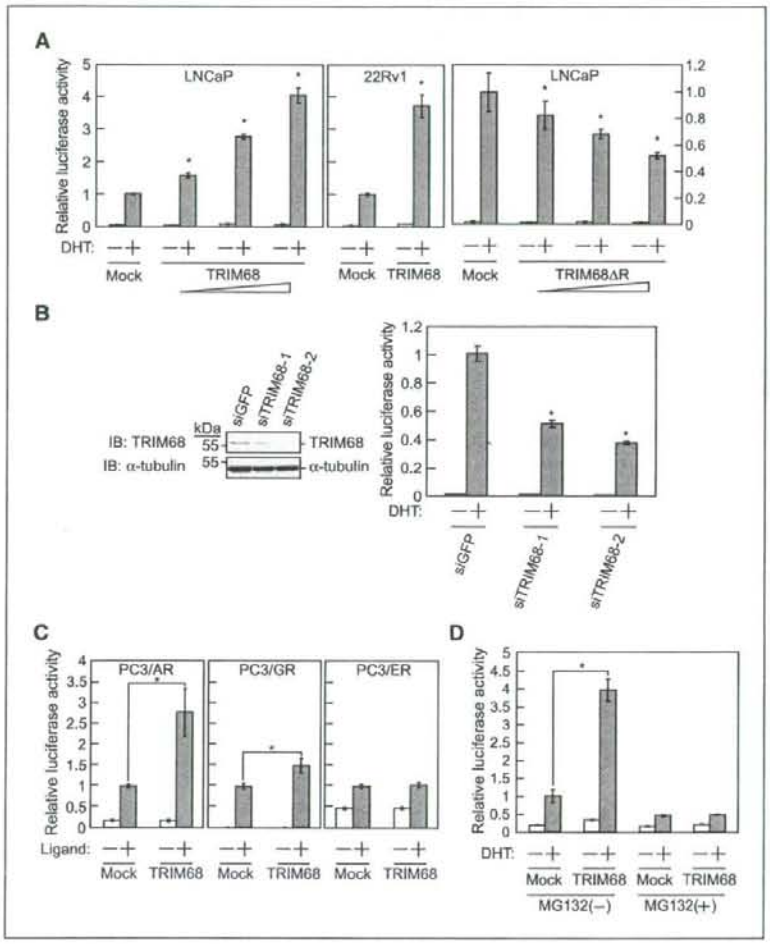

Figure 3. TRIM68 enhances AR-mediated transcriptional activity. *A*, TRIM68 enhances AR-mediated transcriptional activity in a dose-dependent manner. MMTV luciferase reporter vector (MMTV-Luc) and various amounts of TRIM68 expression vector (wild type or ΔR) were transfected into LNCaP (*left and right*) and CWR22Rv1 (*middle*) cells. Transfected cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without 10 nmol/L dihydrotestosterone for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with dihydrotestosterone were defined as 1. *Columns*, mean of values from three independent experiments; *bars*, SD. *, statistically significant based on attaining *P*'s of <0.05 (unpaired Student's *t* test). *B*, knockdown of TRIM68 causes attenuation of AR-mediated transcriptional activity. Two different siRNAs targeting TRIM68 (siTRIM68-1 or siTRIM68-2) or targeting GFP (siGFP, used as a control) were introduced into LNCaP cells by a retrovirus expression system. Knocked-down LNCaP cell lines with siTRIM68 were analyzed by immunoblotting using anti-TRIM68 and anti–α-tubulin (an internal control) antibodies (*left*). Knocked-down LNCaP cell lines with siTRIM68 were transfected with MMTV-Luc reporter vector. Cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without 10 nmol/L dihydrotestosterone for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with siGFP expression vector and then treated with dihydrotestosterone were defined as 1. *Columns*, mean of values from three independent experiments; *bars*, SD (*right*). *C*, effects of TRIM68 on the transcriptional activities of AR, GR, and ER. PC3 cells were transiently transfected with steroid receptor expression vectors (AR, GR, or ER), reporter vectors (MMTV-Luc for AR and GR, ERE-Luc for ER) and TRIM68 expression vector. Transfected cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without a cognate ligand (10 nmol/L dihydrotestosterone, 10 nmol/L dexamethasone, or 10 nmol/L 17β-estradiol) for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with dihydrotestosterone, dexamethasone or 17β-estradiol were defined as 1. *Columns*, mean of values from three independent experiments; *bars*, SD. *D*, proteasome activity is required for the effect of TRIM68 on AR-mediated transactivation. MMTV-Luc and TRIM68 expression vectors were transfected into LNCaP cells. Transfected cells were incubated in 10% charcoal–treated FBS medium for 48 h, treated with 10 μmol/L MG132 for 30 min, and then treated with 10 nmol/L dihydrotestosterone for 8 h. The cells were then harvested and assayed to detect luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with dihydrotestosterone in the absence of MG132 were defined as 1. *Columns*, mean of values from three independent experiments; *bars*, SD.

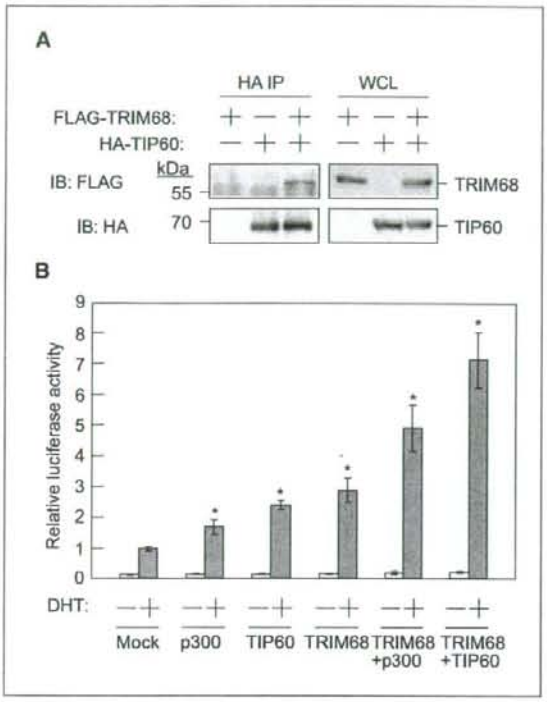

Figure 4. TRIM68 cooperates with TIP60 and p300 to enhance AR-mediated transactivation. *A*, physical interaction between TRIM68 and TIP60. Expression vectors encoding FLAG-tagged TRIM68 and HA-tagged TIP60 were transfected into HEK293T cells as indicated. After 48 h, cells were lysed and subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-FLAG and anti-HA antibodies. Whole-cell lysates were also subjected to immunoblotting to confirm the expression of TRIM68 and TIP60 (*right*). *B*, TRIM68 cooperates with TIP60 or p300 to enhance AR-mediated transactivation. LNCaP cells were cotransfected with expression vectors encoding TIP60, p300, and TRIM68 with MMTV-Luc as a reporter. Transfected cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without 10 nmol/L dihydrotestosterone for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with dihydrotestosterone were defined as 1. *Columns*, mean of values from three independent experiments; *bars*, SD. *, statistically significant based on attaining *P*'s of <0.05 (unpaired Student's *t* test).

TIP60 and/or TRIM68 with MMTV-Luc as a reporter and treated with dihydrotestosterone, and then luciferase assays were performed. TIP60 and TRIM68 individually enhanced AR-mediated transactivation with dihydrotestosterone treatment, and the combination of TIP60 and TRIM68 markedly enhanced AR-mediated transcriptional activity (Fig. 4*B*). Furthermore, the combination of another coactivator, p300, and TRIM68 also enhanced AR-mediated transcriptional activity (Fig. 4*B*). These findings suggest that TRIM68 cooperates with coactivators, including TIP60 and p300, for AR-mediated transactivation.

Effects of TRIM68 on the expression and secretion of PSA. PSA is a secretory glycoprotein that acts as a serine protease and exists exclusively in prostate epithelial cells (27). Serum PSA level is usually increased in patients with prostate cancer (28). Because the *PSA* gene contains an androgen-responsive element (ARE) and its transcriptional level is regulated by AR, we hypothesized that TRIM68 also affects the expression of PSA. LNCaP cells, which express AR and secrete PSA, were used for the PSA expression and secretion assays. LNCaP cells stably expressing TRIM68 or TRIM68ΔRING by a retroviral expression system were treated with or without dihydrotestosterone, and then cell lysates were subjected to immunoblot analysis with anti-PSA antibody (Fig. 5*A*). Furthermore, cell culture supernatants were collected and assayed for PSA concentration by ELISA analysis (Fig. 5*B*). TRIM68 increased both the expression and secretion of PSA by treatment with dihydrotestosterone, whereas TRIM68ΔRING did not. To confirm the relationship between TRIM68 and PSA production, we used LNCaP cells transfected with TRIM68 siRNA and analyzed the expression and secretion levels of PSA. Knockdown of TRIM68 decreased both the expression and secretion of PSA in LNCaP cells (Fig. 5*C* and *D*). These findings suggest that TRIM68 contributes to the expression and secretion of PSA in prostate cancer cells.

TRIM68 is overexpressed in human prostate cancer. Given that TRIM68 modulates AR-mediated transcription, we hypothesized that TRIM68 affects androgen-dependent cell growth. An MTS cell proliferation assay was performed to examine the effects of TRIM68 on cell growth. Knockdown of TRIM68 significantly inhibited the growth of LNCaP cells, whereas overexpression of TRIM68 slightly increased the growth of LNCaP cells (Fig. 6*A*). These results suggest that TRIM68 has a significant effect on the androgen-dependent growth of LNCaP cells.

To examine the effect of TRIM68 on oncogenic phenotype in prostate cancer cells, we performed an anchorage-independent colony formation assay in soft agar. Overexpression of TRIM68 marginally increased colony-forming rate, whereas knockdown of TRIM68 significantly inhibited colony formation of LNCaP cells, indicating that TRIM68 has a significant effect on the oncogenic properties of prostate cancer cells (Fig. 6*B*). These findings may indicate that TRIM68 is required for oncogenic properties of prostate cancer cells but is not sufficient to enhance oncogenic phenotypes (Fig. 6*A* and *B*).

Considering the involvement of TRIM68 in prostate cancer cell proliferation and PSA production, we hypothesized that TRIM68 is aberrantly expressed in human prostate cancers. TRIM68 mRNA levels of 35 cases of human prostate cancer and adjacent normal tissue, which were surgically resected by radical prostatectomy in patients with primary prostate cancer, were quantified by real-time quantitative RT-PCR. TBP was selected as an internal control to normalize the expression levels, because there are no known retropseudogenes for it and TBP is not differentially expressed in tumor and normal prostate tissues (29, 30). Relative mRNA levels of TRIM68 were significantly increased in the majority of human prostate cancers compared with the levels in normal prostate tissues (Mann-Whitney *U* test, $P < 0.05$; Fig. 6*C*). These results indicate that TRIM68 gene expression is up-regulated in human prostate cancer.

Next, to examine the protein expression levels of TRIM68 in human prostate cancers, prostate cancer tissues and adjacent normal tissues simultaneously obtained from radical prostatectomy in patients with primary prostate cancer were analyzed by immunohistochemistry using anti-TRIM68 antibody. TRIM68 staining in tissues was detected mainly in the nucleus of epithelial cells, as observed in LNCaP cells treated with dihydrotestosterone. TRIM68-expressing cells were more abundant in cancer tissues than in normal tissues (Fig. 6*D*, *a–f*). TRIM68 expression in the benign sample group was low or absent (mean staining score, 91). In cancer samples, on the other hand, TRIM68 exhibited mainly a moderate or high level of expression (mean staining score, 220), indicating that TRIM68 immunoreactivity was significantly higher

in cancer tissues than in benign tissues (Fig. 6*D, g*). These findings indicate that TRIM68 is overexpressed in human prostate cancer and may serve as a significant marker protein for prostate cancer.

Discussion

Recent advances have indicated that AR-mediated transactivation is regulated by posttranslational modification, including phosphorylation, acetylation, and ubiquitination. Ubiquitination involves degradation of AR and coregulators, and ubiquitination also has a nonproteolytic role in transcription (10). E3 ubiquitin ligases should play an important role in the regulation of AR-mediated transcriptional activity. However, only a few E3 ligases for AR, coactivators, or corepressors have been identified.

Recently, AR NH_2 terminal–interacting protein, also known as p53-induced protein with a RING-H2 domain (PIRH2), has been reported to interact with histone deacetylace 1 and promote its degradation (31). In addition, E6-AP, a HECT type ubiquitin ligase, has been shown to enhance the hormone-dependent transcriptional activity of AR (32). The coactivating function may result from the ability of E6-AP to target nuclear receptor corepressor (NcoR) for degradation (33). However, PIRH2 and E6-AP do not display tissue-specific expression in prostate, and the effects are not restricted to the regulation of AR activity.

The present study is the first study to provide evidence that TRIM68, which is preferentially expressed in prostate cancer cells, is a novel AR-interacting protein and acts as a coactivator of AR depending on its ubiquitin ligase activity. TRIM68 possesses E3 ubiquitin ligase activity in collaboration with E2, including Ubc4 and UbcH5. TRIM68 physically associates with AR and enhances the transcriptional potential of AR. Interaction of TRIM68 with AR in the nucleus was further enhanced by dihydrotestosterone treatment, indicating that TRIM68 behaves as a coregulator that assembles into an AR-associated transcription factor complex. Overexpression of TRIM68 enhanced AR-mediated transactivation in various prostate cancer cell lines, whereas knockdown of TRIM68 gene expression using RNAi caused suppression of AR-mediated transactivation. These findings indicate that TRIM68 is an intrinsic cofactor for AR activation in prostate cancer cells.

It has been reported that regulation of AR activity by ubiquitination is divided into two roles: a proteolytic role of AR and its coregulators linked to proteasome machinery and a nonproteolytic role without proteasome machinery (34, 35). We showed that the proteasome activity is also required for the effect of TRIM68 on AR-mediated transactivation. Therefore, coactivating function of TRIM68 may be involved in the proteolytic role for AR or AR-associated proteins. However, TRIM68 could not directly ubiquitinate AR (data not shown). Hence, TRIM68 may ubiquitinate one of the corepressors for AR-mediated transcription.

Cyclical recruitment of transcriptional coregulators is now an established phenomenon that is intrinsic to transcriptional activation by steroid receptors (36). However, few molecules that

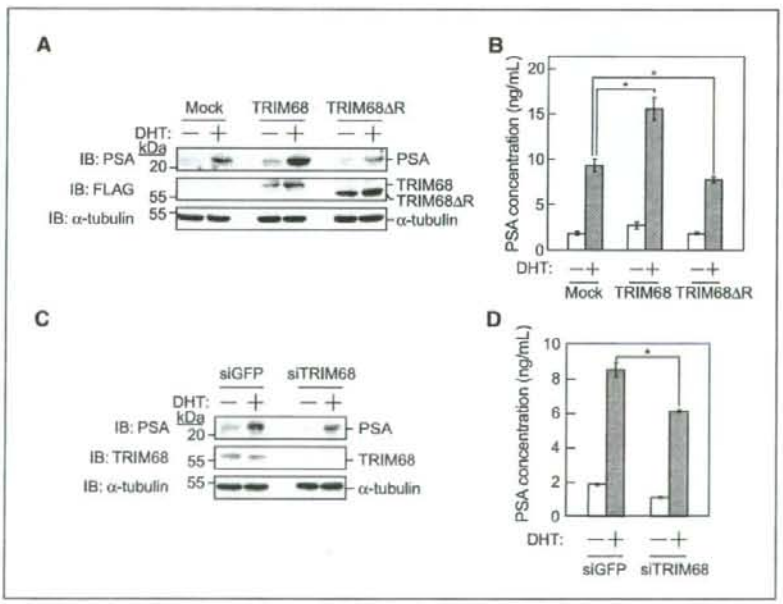

Figure 5. Effects of TRIM68 on PSA expression and secretion. *A*, TRIM68 increases PSA expression in prostate cancer cells. LNCaP cells stably expressing FLAG-tagged TRIM68 or TRIM68ΔR by a retroviral expression system were incubated in 10% charcoal–treated FBS medium for 48 h, washed, and treated with or without 10 nmol/L dihydrotestosterone for 24 h. Cells were lysed and subjected to immunoblotting with anti-PSA, anti-FLAG, and anti–α-tubulin antibodies. *B*, TRIM68 increases PSA secretion in prostate cancer cells. Cell culture media in *A* were collected and assayed for quantifying PSA concentration using ELISA analysis. *Columns*, mean of values from three independent experiments; *bars*, SD. *, statistically significant based on attaining *P*'s of <0.05 (unpaired Student's *t* test). *C*, knockdown of TRIM68 attenuates PSA expression in prostate cancer cells. Stably knocked-down LNCaP cell lines with siTRIM68 or siGFP were incubated in 10% charcoal–treated FBS medium for 48 h, washed, and treated with or without 10 nmol/L dihydrotestosterone for 24 h. Cells were lysed and subjected to immunoblotting with anti-PSA, anti-TRIM68, and anti–α-tubulin antibodies. *D*, knockdown of TRIM68 decreases PSA secretion in prostate cancer cells. Cell culture media in *C* were collected and assayed for quantifying PSA concentration using ELISA analysis. *Columns*, mean of values from three independent experiments; *bars*, SD. *, statistically significant based on attaining *P*'s of <0.05 (unpaired Student's *t* test).

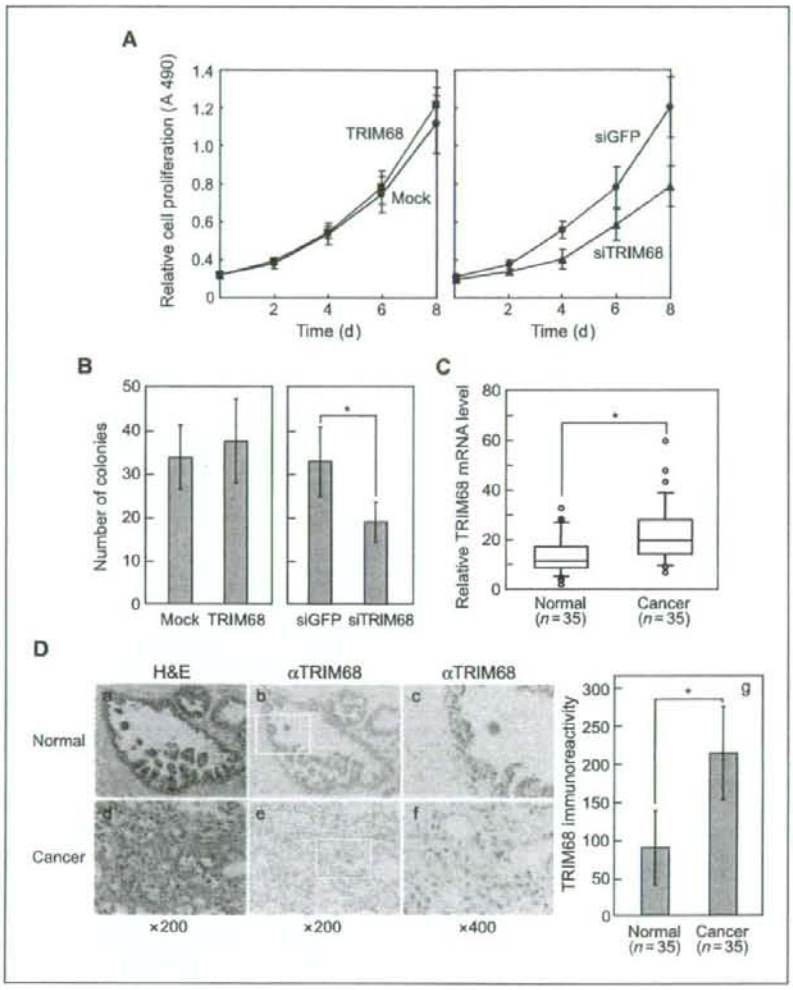

Figure 6. TRIM68 is overexpressed in human prostate cancer. *A*, TRIM68 affects prostate cancer cell growth. LNCaP cell lines stably expressing TRIM68, an empty vector (mock), siTRIM68, or siGFP were incubated in 10% charcoal–treated FBS medium for 48 h and then plated in 96-well plates (5,000 per well). Cells were treated with 10 nmol/L dihydrotestosterone and refed with fresh medium containing dihydrotestosterone every 2 d. Relative cell number was assayed at various times using the MTS assay. Absorbances at 490 nm versus time for each treatment were plotted. *Points*, mean of six replicates; *bars*, SD. *B*, anchorage-independent colony formation assay. Equal numbers of LNCaP cells stably expressing TRIM68, TRIM68 siRNA, or their respective controls were plated in 0.4% soft agar and cultured for 3 wk, and then colonies were counted microscopically. *Columns*, mean of values from three independent experiments; *bars*, SD. *, statistically significant based on attaining *P*'s of <0.05 (unpaired Student's *t* test). *C*, TRIM68 gene expression is up-regulated in human prostate cancer. TRIM68 mRNA levels were compared in human prostate cancers and adjacent normal tissues of 35 cases by real-time quantitative RT-PCR. Samples were surgically resected by radical prostatectomy in patients with primary prostate cancer. The expression level of TRIM68 mRNA was normalized to that of TBP mRNA and shown as relative expression level. The boxes within the plots represent the 25th to 75th percentiles. The horizontal line in the boxes indicates median value. White circles indicate outlier values outside of the 10th and 90th percentiles. *, statistically significant based on attaining *P*'s of <0.05 (Mann-Whitney *U* test). *D*, immunohistochemistry of human prostate tissues with anti-TRIM68 antibody. Samples were surgically resected by radical prostatectomy in patients with primary prostate cancer. Prostate cancer tissues (*d–f*) and adjacent normal tissues (*a–c*) were stained with H&E (*a* and *d*) or with anti-TRIM68 antibody (*b*, *c*, *e*, and *f*). *c* and *f* are higher magnification views of the rectangles in *b* and *e*, respectively. Magnifications, 200× (*a*, *b*, *d*, and *e*) and 400× (*c* and *f*). TRIM68 immunoreactivities were compared in human prostate cancers and adjacent normal tissues of 35 cases by immunohistochemistry (*g*). The immunointensity of samples was categorized as negative (score of 0), weak (score of 1), medium (score of 2), or strong (score of 3), and the final score was obtained by multiplying the percentage of positive cells by the intensity score. *Columns*, mean of values; *bars*, SD. *, statistically significant based on attaining *P*'s of <0.05 (Mann-Whitney *U* test).

regulate the recruitment and activation of coregulators have been identified. We showed that TRIM68 is associated with TIP60 and p300, which act as coactivators of AR, and cooperates in enhancing AR-mediated transcriptional activity. This raises the possibility that TRIM68 assembles into an AR complex with coactivators, such as TIP60 and p300, and ubiquitinate corepressors, leading to exchange of corepressors for coactivators after ligand binding.

The *PSA* gene is known to contain an *ARE*, and its transcriptional level is regulated by AR and coregulators, including TIP60 (37). We showed that TRIM68 increases both expression and

secretion of PSA in LNCaP cells, whereas knockdown of TRIM68 decreases both expression and secretion of PSA. Furthermore, we showed that knockdown of TRIM68 significantly attenuates prostate cancer cell growth. These findings imply that TRIM68 does play an important role in AR transcriptional complexes that regulate gene expression, including expression of the *PSA* gene, or in cell proliferation.

We showed that TRIM68 is predominantly expressed in LNCaP cells among various human cell lines, including sex hormone–related prostate and breast cancer cell lines. Thus, we speculate that effects of TRIM68 are restricted in prostate cancer cells and are particularly involved in AR-mediated transcription. Furthermore, we showed by immunohistochemistry and real-time quantitative RT-PCR that TRIM68 expression is significantly up-regulated in the majority of primary human prostate cancers compared with its expression in adjacent normal prostate tissues. As observed in LNCaP cells with dihydrotestosterone treatment, TRIM68 staining was dominantly detected by immunohistochemistry in the nuclear compartment in human prostate cancer tissues compared with the staining in adjacent normal prostate tissues. These results indicate the possibility that TRIM68 is a potential regulator for prostate carcinogenesis and cancer development.

It has been reported that autoantibodies to TRIM68 are frequently found in the sera of patients with Sjögren's syndrome (19). However, the clinical significance of these autoantibodies in human cancers has not been investigated. Considering the overexpression of TRIM68 in prostate cancers, autoantibodies to TRIM68 may be found in the sera of patients with prostate cancer and could be used as an additional diagnostic tool for prostate cancer.

We showed that TRIM68 is a positive modulator of transcriptional activity of AR. We suggest that ubiquitination activity of TRIM68 regulates the functions of AR or corepressors critically involved in proliferation, differentiation, or oncogenesis of prostate epithelial cells. Thus, it is probably important to identify physiologic substrates of TRIM68 and pharmacologic inhibitors of AR-associated ubiquitin ligases, including TRIM68 for establishing novel therapeutic tools for advanced hormone refractory and metastatic prostate cancer.

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UROLOGY-ORIGINAL PAPER

Early recovery of urinary continence after laparoscopic versus retropubic radical prostatectomy: evaluation of preoperative erectile function and nerve-sparing procedure as predictors

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Abstract The aim of this study was to evaluate preoperative erectile function and attempted nerve-sparing procedure as predictors for early recovery of urinary continence after retropubic and laparoscopic radical prostatectomy. Patients were divided into two groups according to surgical approach (retropubic or laparoscopic) and learning curve for laparoscopic approach: group 1—retropubic approach (37 patients operated on from April 2000 to June 2006), group 2—laparoscopic approach (109 patients operated on from April 2003 to June 2006). We assessed state of urinary continence at 1, 3, 6, and 12 months after removal of the urinary catheter. Overall rates of urinary continence were 18%, 49%, 68%, and 80% at 1, 3, 6, and 12 months, respectively. Between groups 1 and 2, no statistically significant differences in recovery of urinary continence were evident, being 27% versus 15% at 1 month, 54% versus 47% at 3 months, 77% versus 65% at 6 months, and 91% versus 77% at 12 months in groups 1 and 2, respectively. An attempted nerve-sparing procedure (one or both neurovascular bundles) was statistically associated with urinary continence at 3 month, and International Index of Erectile Function-5 (IIEF-5) score ($\geq$14) was identified as a significant factor predicting urinary continence at 6 months after laparoscopic radical prostatectomy. Younger age tended to result in early recovery of urinary continence after retropubic radical prostatectomy.

Keywords Retropubic radical prostatectomy · Laparoscopic radical prostatectomy · Nerve sparing procedure · Preoperative erectile function · Urinary continence

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Introduction

Radical prostatectomy (RP) is a major curative treatment of localized prostate cancer. Functional problems after RP include urinary incontinence and erectile dysfunction (ED). In 1982 Walsh and Donker introduced anatomic retropubic radical prostatectomy (RRP) as a way to decrease postoperative functional complications [1]. Despite progressive improvement over time [2], postprostatectomy urinary incontinence (PPI) persists beyond 1 year in 2–5% of patients, representing a major impairment of quality of life [3]. Factors reported to predict PPI include patient age [4], prostate volume [5], preoperative membranous urethral length according to magnetic resonance imaging [6], and shape of apical prostate [7]. However, predictors for early recovery of urinary continence after RP have not been clearly established.

Nerve-sparing procedure is widely performed to restore erectile function after RP. To determine the adoption of this procedure, preoperative erectile function is assessed in addition to tumor stage. Recently, some authors have described the efficacy of nerve-sparing procedure for recovery of urinary continence [8–10], and others have concluded that preoperative ED was one of the predictors for persistent PPI [11].

Since 1997, laparoscopic radical prostatectomy (LRP) has been widely performed because of its minimal invasiveness. However, likelihood of urinary continence after LRP has been little better than after RRP [12]. Since 2000 we have used the laparoscopic approach for RP. Our preliminary functional assessments found no difference between LRP and RRP in terms of urinary continence 12 months after surgery [13]. In the present study we compared continence rate in the early period after surgery between RRP and LRP, and evaluated both preoperative erectile function and an attempted nerve-sparing procedure as predictors of early recovery of strictly defined urinary continence.

Patients and methods

From April 2000 to June 2006, we performed RP in 241 consecutive men with prostate cancer at Kobe University Hospital, Japan, using the retropubic or laparoscopic approach. The retropubic approach was performed as described by Walsh [14], with certain modifications. The laparoscopic approach was performed as reported originally by the Montsouris group [15]. All patients were continent for urine before RP. Patients receiving neoadjuvant hormonal therapy or preoperative radiotherapy were excluded from this analysis. Those with postoperative anastomotic stricture or rectourethral fistulas were also excluded from this study. Furthermore, 64 patients who underwent LRP from April 2000 to March 2003 were excluded from this study in terms of learning curve for LRP. In all, 146 patients (RRP 37, LRP 109) remained eligible for assessment of urinary continence after catheter removal. Both in RRP and in LRP, the urinary catheter was removed at 7 days after surgery unless urinary leakage from the vesicourethral anastomosis persisted.

Patients were divided into two groups according to procedure: group 1-RRP (37 patients), group 2-LRP (109 patients performed between April 2003 and June 2006). State of preoperative erectile function was assessed using the International Index of Erectile Function-5 (IIEF-5) questionnaire before surgery [16]. No patient has received phosphodiesterase-5 inhibitors before surgery. In each group, urinary continence was also compared between the patients with IIEF-5 score <14 and those with IIEF-5 score ≥ 14. Indication for nerve-sparing procedure depended on preoperative and intraoperative factors. Bilateral nerve-sparing procedure was offered to patients with prostate-specific antigen (PSA) <10 ng/ml, Gleason score of ≤ 7, and location of biopsy of cancer positive specimen not close to the neurovascular bundle (NVB). Unilateral nerve-sparing procedure was offered to patients when one side of the apex was free of cancer and no more than one biopsy was positive on the ipsilateral side. The patients were mailed a questionnaire regarding the number of sanitary pads used 24 h preoperatively and postoperatively. Urinary continence was defined as no pad requirement over 24 h. The primary outcome of interest was state of continence at 1, 3, 6, and 12 months after removal of the urinary catheter.

The Mann–Whitney test was used for quantitative variables, and the chi-square test was used for categorical variables. Multivariate logistic regression analysis was performed to investigate the association between urinary continence and patient age at surgery, preoperative IIEF-5 score (≥ 14), and an attempted nerve-sparing procedure (one or both NVBs). A difference was considered statistically significant when the p value was less than 0.05. Statistical analyses were performed using StatView 5.0 (SAS Institute INC., Cary, North Carolina) software.

Results

Patient characteristics are shown in Table 1. Statistically significant differences between two groups were found in the distribution of clinical T stage, Gleason sum obtained from biopsy specimens, and prostate volume.

Overall rates of urinary continence were 18%, 49%, 68%, and 80% at 1, 3, 6, and 12 months, respectively. Changes over time for rate of urinary continence in each group are shown in Fig. 1. In group 1, rate of urinary continence was 27%, 54%, 77%, and 91% at 1,

Table 1 Preoperative/baseline patient characteristics

	Group 1 ($n = 37$)	Group 2 ($n = 109$)	P-value
Age at surgery, years (mean ± SD)	67.1 ± 6.0	66.1 ± 6.3	0.4424
BMI, kg/m^2 (mean ± SD)	23.5 ± 3.0	23.8 ± 2.5	0.3945
PSA, ng/ml (mean ± SD)	14.7 ± 11.9	11.0 ± 8.4	0.0635
Clinical T stage			0.0121
T1c	6	41	
T2a	11	16	
T2b	11	28	
T2c	2	16	
T3a	6	8	
T3b	1	0	
Preoperative IIEF-5 score (mean ± SD)	9.6 ± 7.3	10.1 ± 7.8	0.8105
Gleason score (sextant biopsy) (mean ± SD)	6.9 ± 1.0	6.6 ± 0.7	0.0473
Prostate volume (ml) (mean ± SD)	30.1 ± 26.9	32.2 ± 16.5	0.0299
Neurovascular preservation (number of patients)			0.0851
Attempted bilateral	0	11	
Attempted unilateral	13	43	
Without attempted	24	55	

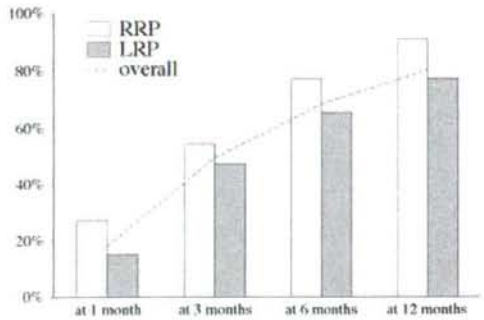

Fig. 1 Continence rate at 1, 3, 6, and 12 months after radical prostatectomy. Group 1, retropubic; group 2, laparoscopic approach, between April 2003 and June 2006. No statistical significances in continence rate at each time between the two groups were found, but continence rate at 1 and 12 months in group 1 tended to be higher than in group 2 ($P = 0.0998$, $P = 0.4675$, $P = 0.1748$, and $P = 0.0577$ at 1, 3, 6, and 12 months, respectively)

3, 6, and 12 months, respectively. In group 2, rate of urinary continence was 15%, 47%, 65%, and 77% at 1, 3, 6, and 12 months, respectively. No statistically significant differences were found between these two groups.

Univariate analyses are shown in Figs. 2 and 3. In group 1 (Fig. 2), age at surgery was associated with the recovery of urinary continence at 1 and 3 months ($P = 0.0199$ and $P = 0.0107$, respectively). Attempted nerve-sparing procedure (one or both NVBs) was associated with recovery of urinary continence at 6 months ($P = 0.0316$), however a significant association between preoperative IIEF-5 score ($\geq$14) and the recovery of urinary continence was not shown in group 1. In group 2 (Fig. 2), attempted nerve-sparing procedure was associated with urinary continence at 1, 3, and 6 months ($P = 0.0323$, $P = 0.0335$, and $P = 0.0090$, respectively). Furthermore, preoperative IIEF-5 score ($\geq$14) was associated with urinary continence at 6 months ($P = 0.0475$). At 12 months postoperatively, there were no statistically significant differences between urinary continence and each parameter in these two groups.

In multivariate analyses including patient age, preoperative IIEF-5 score ($\geq$14), and attempted nerve-sparing procedure (one or both NVBs), age at surgery was not identified as an independent factor for early recovery of urinary continence, except that it was marginally significant at 1 and 3 months in group

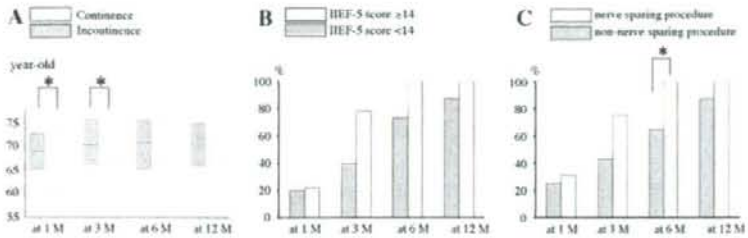

Fig. 2 Correlation between urinary continence and each parameter in group 1 (retropubic approach). (**a**) Distribution of age at surgery in continent and incontinent patients at 1, 3, 6, and 12 months. (**b**) Continence rates of IIEF-5 score ≥14 group and <14 group at 1, 3, 6, and 12 months. (**c**) Continence rates of nerve-sparing procedure and non-nerve-sparing procedure groups at 1, 3, 6, and 12 months. Age at surgery was statistically associated with recovery of urinary continence at 1 and 3 months, and nerve-sparing procedure was also associated with recovery of urinary continence at 6 months. M, months; $^*P < 0.05$

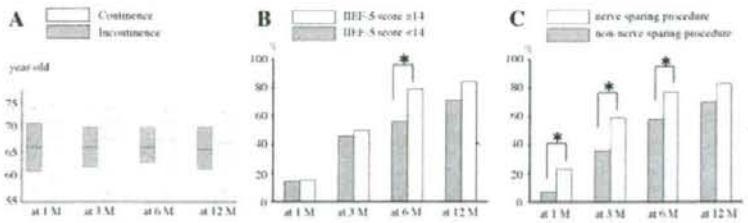

Fig. 3 Correlation between urinary continence and each parameter in group 2 (laparoscopic approach, between April 2003 and June 2006). (**a**) Distribution of age at surgery in continent and incontinent groups at 1, 3, 6, and 12 months. (**b**) Continence rate of IIEF-5 score ≥14 group and <14 group at 1, 3, 6, and 12 months. (**c**) Continence rate of nerve-sparing procedure and non-nerve-sparing procedure group at 1, 3, 6, and 12 months. IIEF-5 score was associated with recovery of urinary continence at 6 months. Nerve-sparing procedure was also associated with recovery of urinary continence at 1, 3, and 6 months. M, months; $^*P < 0.05$

Table 2 Multivariate analyses using logistic regression test (group 1; RRP)

		P-value	OR	95% CI
At 1 month	Age at surgery	0.0682	0.813	0.651–1.016
	IIEF-5 score ≥14	0.6099	0.514	0.040–6.630
	Attempted nerve-sparing procedure	0.6105	1.896	0.162–22.222
At 3 months	Age at surgery	0.0524	0.794	0.629–1.002
	IIEF-5 score ≥14	0.2449	3.529	0.421–29.560
	Attempted nerve-sparing procedure	0.2630	3.655	0.378–35.359
At 6 months	Age at surgery	0.1124	0.745	0.518–1.071
	IIEF-5 score ≥14	0.9966	Not available	Not available
	Attempted nerve-sparing procedure	0.9963	Not available	Not available

1 ($P = 0.0682$ and $P = 0.0524$, respectively; Table 2). In group 2 (Table 3), nerve-sparing intent was statistically associated with urinary continence at 3 months, and marginally significant at 1 month ($P = 0.0350$ and $P = 0.0565$, respectively). At 6 months, urinary continence was not associated with nerve-sparing procedure statistically, but was with preoperative IIEF-5 score (≥14) ($P = 0.0422$).

Table 3 Multivariate analyses using logistic regression test (group 2; LRP)

		P-value	OR	95% CI
At 1 month	Age at surgery	0.3711	1.051	0.942–1.173
	IIEF-5 score ≥14	0.8766	1.113	0.290–4.275
	Attempted nerve-sparing procedure	0.0565	3.915	0.963–15.922
At 3 months	Age at surgery	0.5670	1.022	0.949–1.100
	IIEF-5 score ≥14	0.8079	1.126	0.433–2.927
	Attempted nerve-sparing procedure	0.0350	2.609	1.070–6.363
At 6 months	Age at surgery	0.6529	1.018	0.941–1.102
	IIEF-5 score ≥14	0.0422	3.047	1.040–8.930
	Attempted nerve-sparing procedure	0.1369	2.069	0.794–5.392

Discussion

PPI is a major concern for many patients undergoing RP. Sphincteric weakness is a cause of PPI [17, 18], and functional urethral length also influences urethral closure pressure [19]. PPI affects 30–85% of patients for a variable period between 3 weeks and 6 months [3, 12]. At 12 months, the rate of urinary continence varies from 89% to 98% after RRP [8, 20–22], and from 84% to 97% after LRP [15, 21, 23]. Although LRP is typically chosen because it is a less invasive procedure than RRP, functional outcome concerning urinary continence after LRP has not been superior to that after RRP [12]. In agreement, our limited experience revealed no significant difference between urinary continence after RRP and that after LRP.

LRP has been widely performed only for a decade. Guillonneau and Vallancien reported that the "learning curve" affected about 40 cases per surgeon owing to various operative steps carried out during LRP [15]. Although Rassweiler et al. reported that surgeon experience did not affect urinary continence at 12 months after RRP, level of expertise significantly impacted early recovery of urinary continence [21]. In the present study, continence rate at 3 months after LRP performed before April 2003 was worse than that for LRP performed in subsequent months (data not shown). The learning curve might influence early recovery of urinary continence after LRP. We therefore excluded patients who underwent LRP between April 2000 and March 2003 from the analyses in this study.

Our study showed that preoperative ED was significantly associated with PPI at 6 months after late-period LRP. Peripheral vascular disease is an important cause of vasculogenic ED [24]. The blood supply to the penis is ordinarily provided the internal pudendal artery and the accessory pudendal artery [25]. Similarly, the blood supply to the membranous urethra is mainly provided by the internal pudendal artery [26]. John et al. reported that membranous urethral blood flow significantly decreased after RRP [27]. During laparoscopic surgery, abdominal CO_2 insufflation in pneumoperitoneum and the subsequent increase in abdominal pressure can cause severe changes in hemodynamics. Venous compression by the positive pressure of CO_2 insufflation during LRP might decrease blood supply to the membranous urethra beyond any decrease occurring during RRP. Decreased blood flow to the membranous urethra, even if it is temporary, might result in urethral sphincteric insufficiency in some patients with preoperative vasculogenic ED. However, preoperative ED in late-period LRP cases was not associated with urinary incontinence at 1 and 3 months. Urinary continence at these time points might be influenced by nonvascular factors, such as trigonal denervation and periurethral fibrosis [28].

Although benefits of nerve-sparing procedures in maintaining erectile function have been widely emphasized, the contribution of such procedures to urinary continence after RP is controversial [2, 3, 5, 8, 10, 11]. The external urethral sphincter is innervated by both autonomic nerve fibers from the pelvic plexus and somatic nerve fibers from the pudendal nerves [29, 30]. We previously reported findings based on intraoperative electrical stimulation that indicated that the NVB contained nerves supplying the membranous urethra [31]. These anatomic and clinical observations suggested that compromise of

the NVB during RP could influence postoperative urinary continence. In the present study, attempted nerve-sparing procedure was significantly associated with urinary continence. However, the number of patients with an attempted bilateral nerve-sparing procedure was smaller in our series than in some other reports. This could explain why multivariate analyses identified attempted nerve-sparing procedure as a significant factor for recovery of urinary continence only at 3 months. We also recognize that our study had limitations due to the number of operations performed using nerve-sparing procedure and the learning curve of LRP. Despite these limitations our results suggest that preoperative erectile function and attempted nerve-sparing procedure influenced the status of postoperative urinary continence.

In conclusion, no significant differences in early recovery of urinary continence were found between LRP performed by an experienced surgeon and RRP. Preoperative erectile function and nerve-sparing procedure predicted early recovery of urinary continence after LRP by an experienced surgeon. Similarly to other reports [2, 22], patient age at surgery tended to influence early recovery of urinary continence after RRP in our limited series.

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Haptoglobin-β chain defined by monoclonal antibody RM2 as a novel serum marker for prostate cancer

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In our previous study, monoclonal antibody RM2, established toward the glycosyl epitope, reflected grade of malignancy of prostate cancer cells whereas RM2 reactivity to benign glands was negative or weak. RM2 reactivity was also detected in stroma, suggesting the glycoprotein RM2 recognizes could be released into the bloodstream. Then, we explored RM2 reactivity to sera of early prostate cancer. We compared RM2 reactivity to sera between 62 patients with early prostate cancer and 43 subjects with benign prostatic disease, and examined RM2 reactivity before and after radical prostatectomy in 15 patients by Western blotting. We also examined RM2 reactivity to sera of the other urogenital cancers. RM2 reactivity was significantly enhanced on a serum glycoprotein with molecular mass ~40 kDa, hereby termed GPX, in the patients with early prostate cancer when compared with those with benign prostatic disease ($p < 0.0001$). Setting an appropriate cutoff level, RM2 reactivity to GPX for detection of prostate cancer had sensitivity of 87% and specificity of 84%, respectively. Furthermore, the level of RM2 reactivity significantly decreased after radical prostatectomy ($p = 0.006$). However, increased RM2 reactivity to GPX was also observed in the other urogenital cancers. The proteomics approach identified GPX as haptoglobin-β chain and RM2 showed preferential reactivity toward haptoglobin-β chain derived from prostate cancer when compared with polyclonal anti-haptoglobin antibody. Haptoglobin-β chain defined by RM2 is a novel serum marker that may be useful for detection of early prostate cancer when coupled with prostate-specific antigen because it is not specific to prostate cancer.

Key words: prostate cancer; haptoglobin; serum marker; RM2

Prostate-specific antigen (PSA) has a problem especially with specificity, *i.e.*, PSA is not only elevated in prostate cancer but also in benign prostatic disease (BPD). Thus, only 25% of men with PSA value of 4 to 10 ng/mL will have diagnosis of cancer after prostate biopsy.[1–3] Furthermore, PSA alone cannot predict pathological stage of prostate cancer because PSA does not reflect the grade of malignancy.[1–4] Recently, it was pointed out that the prevalence of prostate cancer was 15% among men with PSA 4 ng/mL or less.[4] Therefore, a new serum marker to compensate for the problems of PSA is urgently required. However, there has been few serum markers reported, which have potential for clinical application.[5,6] We previously reported that monoclonal antibody (mAb) RM2 was established toward disialoganglioside and later found to recognize the glycosyl epitope (β1.4-GalNAcDSLc4).[7,8] In an attempt to find a new marker, we examined whether immunoreactivity of mAb RM2 was detected in radical prostatectomy specimens, and found that reactivity of mAb RM2 to prostate cancer cells was associated with grade of malignancy, whereas RM2 reactivity to benign glands was negative or weak.[9] RM2 immunoreactivity was also detected in stroma,[9] suggesting the glycoprotein RM2 recognizes may be shed from cancer cells into the surrounding stroma and then released into the bloodstream. In the current study, we explored RM2 reactivity to sera of early prostate cancer, and found that mAb RM2 also recognized haptoglobin-β chain and level of haptoglobin-β chain defined by RM2 significantly increased in sera of early prostate cancer.

Subjects and methods

Serum samples

Of serum samples obtained from Department of Urology, Tohoku University Hospital between June 2004 and May 2006, serum samples of 62 patients with early prostate cancer and those of 43 with BPD were randomly selected. All these subjects had biopsy proven histological diagnosis and PSA less than 10 ng/mL. Fifteen serum samples of the patients with various PSA values who underwent radical prostatectomy, 6 of the subjects with renal cell carcinoma (RCC), 8 of those with urothelial carcinoma and 8 of those with testicular germ cell tumors were also randomly selected. The Ethics Committees of both Tohoku University Graduate School of Medicine and Pacific Northwest Research Institute approved the present study, and informed consent was obtained from each patient. Clinical tumor-node metastasis staging was assigned using the 1997 tumor-node metastasis staging system.[10] Gleason scores of all slides were diagnosed by a single pathologist (M.E.).[11]

Cell lines

Prostate cancer cell lines PC3, LNCaP and DU145 were obtained from Human Science Research Resource Bank (Wako, Japan). Androgen-independent prostate cancer cell line AlCaP1 was newly established from LNCaP in our laboratory (Taima

Abbreviations: BPD, benign prostatic disease; CBB, coomassie brilliant blue; IEF, isoelectric focusing; mAb, monoclonal antibody; PCa, prostate cancer; RCC, renal cell carcinoma; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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et al., to be published elsewhere). PrEC, normal human prostatic epithelial cells were purchased from Cambrex Bioscience (Walkersville, MD). RCC cell line ACHN was purchased from Dainihonseiyaku. (Osaka, Japan) and TOS1 was previously established in our laboratory.[7]

Antibodies

mAb RM2 (isotype IgM) was established using RCC TOS1 cells as immunogen,[7] based on our earlier studies that indicated that degree of RCC malignancy was correlated with disialoganglioside expression in RCC cells.[12] RM2 is reactive with disialoganglioside but not with monosialoganglioside fraction, and the antigen was later identified as β1,4-GalNAc-disialyl-Lc4.[8] Polyclonal anti-haptoglobin antibody was purchased from Dako (Dako Cytomation Kyoto, Japan).

Western blotting of serum

After removing albumin and IgG using Aurum™ Serum Protein Mini Kit (Bio-Rad), a 20 μl aliquot of serum was electrophoresed on an 10% SDS-PAGE and transferred to Hybond P PVDF membrane (Amersham Biosciences, Uppsala, Sweden). Immunoblotting was performed as described previously.[9] Densitometric analysis of RM2 reactivity to a glycoprotein with molecular mass ~40 kDa (GPX) in serum was performed using Scion image (Scion Corp., Frederick, MD) and each value of ~40 kDa glycoprotein was normalized to that of a glycoprotein with molecular mass ~75 kDa from the same lane.

Pretreatment of sera by Agilent column, followed by 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE)

Agilent Multiple Affinity Removal System 1.6 × 100 mm^2 column, designed to adsorb >98–99% of 6 abundant proteins (albumin, immunoglobulins IgG and IgA, transferrin, haptoglobin and antitrypsin) from human serum samples, was purchased from Agilent Technologies (Palo Alto, CA) together with solvents A and B used for adsorption and elution of the proteins.

Two-dimensional SDS gel electrophoresis, *in situ* alkylation, Western blotting and *in gel* digestion were performed as described previously.[13]

Identification of protein

The tryptic digest was analyzed using Agilent 110 capillary HPLC (Agilent Technologies) combined with LCQ ion trap mass spectrometer (Thermo Electron, Waltham, MA). Data were searched against NCBI human sequence database using TurboSEQUEST v.27, and in selected cases Mascot (Matrix Science).

mRNA levels of haptoglobin-β chain in prostate cancer cell lines

Total RNA was extracted from PC3, LNCaP, DU145 and PrEC using Trizol reagent (Gibco BRL, Grand Island, NY) following the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed into first-strand cDNA using ExScript reverse transcription kit (Takara Bio, Japan). PCR was performed using the primers of haptoglobin-β chain and those of β-actin.

Immunohistochemical staining

RM2 reactivity to prostate cancer was described in our previous study.[9] Twelve cases of benign prostatic hyperplasia were immunostained by mAb RM2 in this study. To examine haptoglobin expression in prostate cancer, polyclonal anti-human haptoglobin antibody was used because antibody specific to haptoglobin-β chain was not available. Twenty cases of radical prostatectomy specimens as previously described[9] were immunostained by polyclonal anti-haptoglobin antibody. Immunostaining intensity was graded as negative, weak and moderate to strong. Then, immunoreactivity of each slide was either classified as lower expression or higher expression based on staining intensity and percentage of cancer cells stained. When moderate to strong staining was observed in 10% or more of cancer cells, higher expression was assigned, and when moderate to strong staining was observed in less than 10% of cancer cells, lower expression was assigned.

Extraction of glycosphingolipids and thin-layer chromatography immunostaining

We investigated whether GalNAcDSLc4 as a ganglioside is also responsible for RM2 reactivity to prostate cancer cells. Briefly, glycosphingolipids were extracted from prostate cancer cell lines and separated from phospholipids by alkaline degradation of phospholipids, and glycosphingolipids were analyzed by thin-layer chromatography with immunostaining by mAb RM2 as described previously.[14] To examine whether GalNAcDSLc4 as a ganglioside was released into the culture media, glycosphingolipids were extracted from 10 ml of supernatant of cell lines cultured in serum-free media for 3 days by solid phase extraction using C18 Sep-Pak cartridge (Waters, Milford, MA) as described previously.[15] Monosialosyl globopentaosylceramide (MSGb5) from supernatant of ACHN cells was used as a positive control.

Treatment of protein extract from prostate cancer cells with the hemoglobin column and immunoblotting by mAb RM2

We examined whether RM2 reactivity to prostate cancer cells depends on haptoglobin-β chain because GalNAcDSLc4 as a ganglioside was not detected in prostate cancer cells as described in Results section. For this purpose, protein extract from prostate cancer cell line DU145 was treated with the hemoglobin column to adsorb haptoglobin. DEAE-purified human hemoglobin was purchased from Sigma and coupled to CNBr-activated Sepharose-4B (Sigma) according to the manufacturer's procedures. One hundred microliters of protein extract of DU145 was applied to the column and the eluate (termed fraction I) was immediately collected without incubation. Another 100 μl was reacted with the hemoglobin column beads at 4°C overnight, then the column was stood for 5 min, and the eluate (termed fraction II) passed through the column was collected. After collecting the fraction II, the column was washed with PBS and the washed fraction (termed wash I) was collected. RM2 reactivity to each fraction was examined by Western blotting.

Treatment of serum haptoglobin-β chain defined by mAb RM2 with glycosidase

Although our coworkers recently indicated that RM2 glycosyl epitope (β1.4-GalNAcDSLc4) was not found on haptoglobin-β chain,[16] we examined changes of RM2 reactivity after β-hexosaminidase and/or sialidase treatment on the assumption that RM2 reactivity to haptoglobin-β chain is based on the RM2 glycosyl epitope. After transferring proteins from the gel to the membrane, the membrane was blocked with 1% bovine serum albumin solution at room temperature for 2 hr and washed with phosphate buffered saline/0.05% Tween-20. The membrane was placed into a vinyl bag and treated with 2.0 U of β-hexosaminidase from jack beans (Sigma) at 37°C overnight or with 25 mU of sialidase from Newcastle disease virus (NDV; Glyko) at 37°C overnight. The membrane was washed with phosphate buffered saline/0.05% Tween-20 and subjected to Western blotting by mAb RM2. The control membrane was treated in the same way without glycosidase.

Statistical analysis

Statistical analysis was performed using the software from the SAS Institute (SAS Institute, Cary, NC).

Results

RM2 reactivity to sera of prostate cancer and BPD

For comparison with RM2 reactivity to serum, examples of RM2 reactivity to prostate cancer cells and to benign prostatic

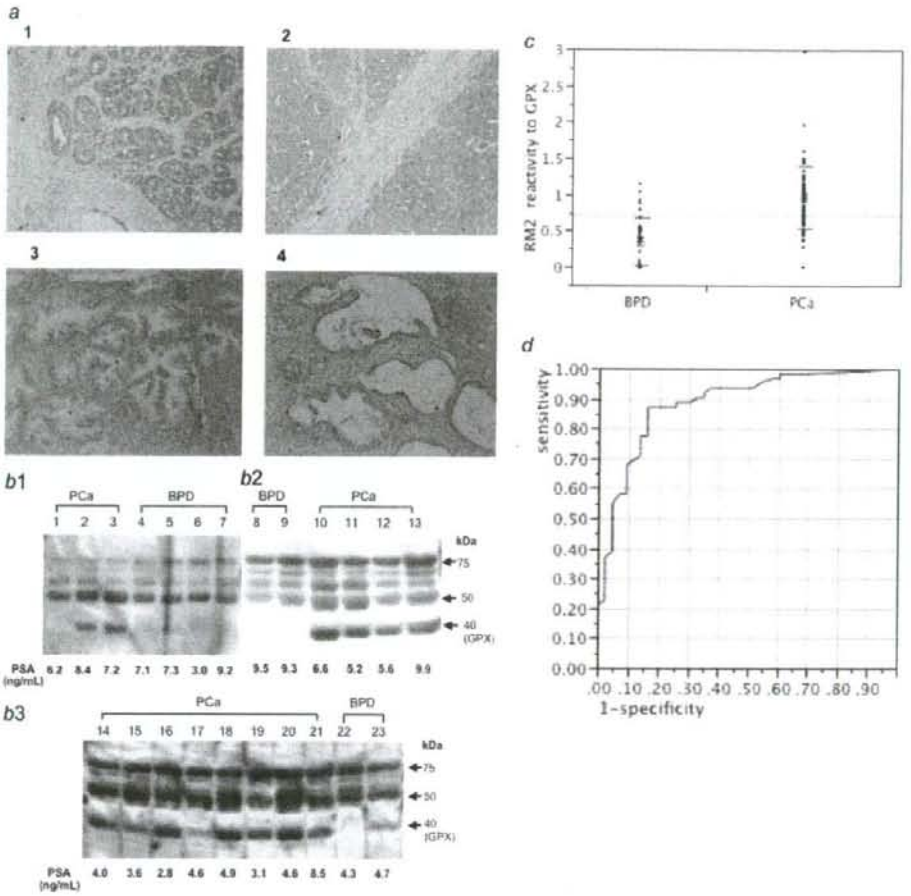


FIGURE 1 – Increased level of RM2 reactivity to GPX in sera of prostate cancer. (Panel *a*) Examples of immunohistochemical staining of prostate cancer cells (panels 1 and 2) and benign prostatic hyperplasia (panels 3 and 4). RM2 reactivity to stroma was also observed in prostate cancer tissues, but neither gland nor stroma in benign prostatic hyperplasia was stained by mAb RM2. (Panel *b*) Examples of serum Western blotting by mAb RM2. PCa: prostate cancer. BPD: benign prostatic disease. Arrow indicates position of size marker and GPX. In each panel, PSA level for each case was indicated at bottom. (Panel *c*) Comparison of RM2 reactivity to GPX between BPD and PCa. Large bars: standard deviation, Small bars: standard error of the mean. (Panel *d*) ROC curve of RM2 reactivity to GPX. The area under the ROC curve was 0.89. The difference between sensitivity and 1-specificity was highest at sensitivity of 87%.

hyperplasia were shown in Figures 1*a* and 1*b*, respectively. None of the 12 cases of benign prostatic hyperplasia examined was stained by mAb RM2. Level of RM2 reactivity to prostate cancer was associated with grade of malignancy as previously described.[9]

We found that RM2 reactivity was enhanced on a serum glycoprotein with molecular mass ~40 kDa, hereby termed "GPX," in the majority of 62 patients with early prostate cancer, when compared with 43 subjects with BPD (Figs. 1*b* and 4*b* right panel). No reactivity was observed for mouse IgM used as a negative control (data not shown). All these subjects had biopsy proven histological diagnosis and PSA less than 10 ng/mL (Tables I and II). There was no significant difference of age and PSA between the 2 groups. RM2 reactivity to GPX calculated by Scion image was normalized to the reactivity to ~75 kDa protein, which was used as an internal control because RM2 reactivity to ~75 kDa protein was relatively constant between prostate cancer and BPD. Level of RM2 reactivity to GPX in prostate cancer (0.96 ± 0.43) was significantly higher than that in BPD (0.35 ± 0.32) ($p < 0.0001$ by t test) (Fig. 1*c*). The receiver operating characteristic analysis was performed and the area under the receiver operating characteristic curve of RM2 reactivity to GPX was 0.89. Setting a cutoff level of RM2 reactivity to GPX as 0.59, RM2 reactivity to GPX for detection of prostate cancer had sensitivity of 87% and specificity of 84% (Fig. 1*d*). Then, we explored the variables predicting the level of RM2 reactivity to GPX by univariate analysis. Level of RM2 reactivity to GPX was not significantly associated with the pretreatment variables in 62 subjects with early prostate cancer (Table III). It was significantly associated with the origin of index (major) cancer among the postsurgical variables in 24 patients, who underwent radical prostatectomy (Table IV), *i.e.*, the index cancer of transition zone origin predicted lower level of RM2 reactivity when compared with that of peripheral zone origin.

TABLE I – CLINICAL CHARACTERISTICS OF THE PATIENTS WITH BIOPSY PROVEN HISTOLOGICAL DIAGNOSIS AND PSA VALUE LESS THAN 10 NG/ML

	PCa	BPD	*p*
Age	68.6 ± 6.4	66.8 ± 7.6	0.2
PSA	5.3 ± 2.1	5.0 ± 2.1	0.4
F/T	0.16 ± 0.1	0.22 ± 0.1	0.007

PCa, prostate cancer; BPD, benign prostatic disease; F/T, ratio of free to total PSA.

TABLE II – CLINICOPATHOLOGICAL CHARACTERISTICS OF THE PROSTATE CANCER PATIENTS WITH PSA VALUE LESS THAN 10 NG/ML

Parameters	No. Patients
cT	
Ib	1
Ic	45
2a	11
2b	4
3a	1
bGS	
6	8
7	43
8	5
9	6

cT, clinical T stage; bGS, biopsy Gleason score.

TABLE III – THE RELATIONSHIP BETWEEN THE PRETREATMENT VARIABLES AND RM2 REACTIVITY

Variables	*p*
Age	0.1769
PSA	0.0922
Bgs	0.4023
CT	0.8196
No. positive biopsy core	0.1429

cT, clinical T stage; bGS, biopsy Gleason score.

TABLE IV – THE RELATIONSHIP BETWEEN THE POSTSURGICAL VARIABLES AND RM2 REACTIVITY

Variables	*p*
Age	0.0980
PSA	0.9843
RPGS	0.3723
Index cancer origin	0.0117
Total cancer volume	0.3433
pT	0.6099

RPGS, radical prostatectomy Gleason score; pT, pathological T stage.

RM2 reactivity to GPX before and after radical prostatectomy was also examined in 15 patients with various preoperative PSA levels whose serum PSA level decreased to less than 0.1 ng/mL after radical prostatectomy, the value believed to be recurrence-free (Table V). Level of RM2 reactivity to GPX decreased in 13 of these 15 patients after radical prostatectomy, although the extent of decrease was varied (Figs. 2*a* and 2*b*). Level of RM2 reactivity significantly decreased after radical prostatectomy (pre- *vs.* post-operative value: 0.92 ± 0.52 *vs.* 0.60 ± 0.43; $p = 0.006$ by paired *t* test) (Fig. 2*b*). The profile of RM2 reactivity to sera of the other urogenital malignancies was almost the same as that to sera of prostate cancer, and level of RM2 reactivity to GPX 0.59 or more was observed in 5 of 6 subjects with RCC, 6 of 8 with urothelial carcinoma, and 2 of 8 with testicular germ cell tumor (data not shown), *i.e.*, RM2 reactivity to GPX was not specific to prostate cancer.

Identification of GPX as haptoglobin-β chain

In view of the clinical significance of GPX, we analyzed its molecular parameters. GPX was separated by Agilent column, followed by application of proteomics approach with 2D gel electrophoresis, in gel digestion, HPLC and electrospray ionization mass spectrometry.[13] The results showed clearly that GPX is haptoglobin-β chain (Fig. 3). In 2D SDS-PAGE, a clear difference was found in one fraction consisting of four contiguous spots (termed spot 1, 2, 3 and 4 from right to left), from malignant sera (specimen II-c: Fig. 3*a-1*, upper panel) *vs.* corresponding spots from nonmalignant sera (specimen II-b: Fig. 3*a-1*, lower panel). The difference between nonmalignant *vs.* malignant sera was more distinct when analysis was made by 2D SDS-PAGE with Western

TABLE V – CLINICOPATHOLOGICAL CHARACTERISTICS OF THE PATIENTS WHOSE RM2 REACTIVITY WAS COMPARED BEFORE AND AFTER RADICAL PROSTATECTOMY

Age (median)	55–75 yr (67)
Preop. PSA (median)	3.07–24.29 ng/ml (5.41)
Pathological parameters	No. patients
pT	
2a	1
2b	9
3a	5
RPGS	
5	1
7	13
8	1

Preop., preoperative; pT, pathological T stage; RPGS, radical prostatectomy Gleason score.

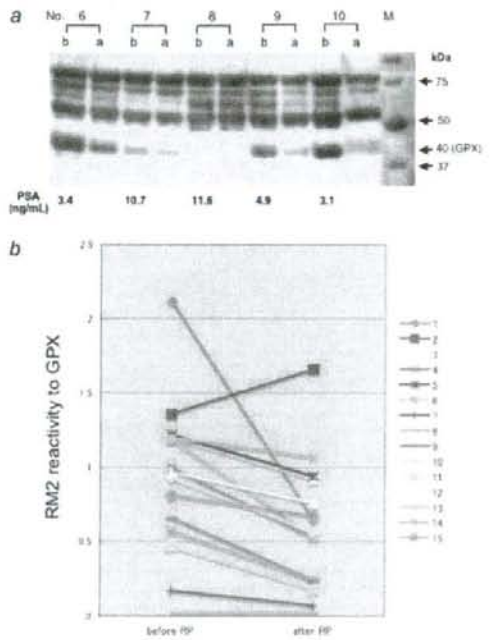
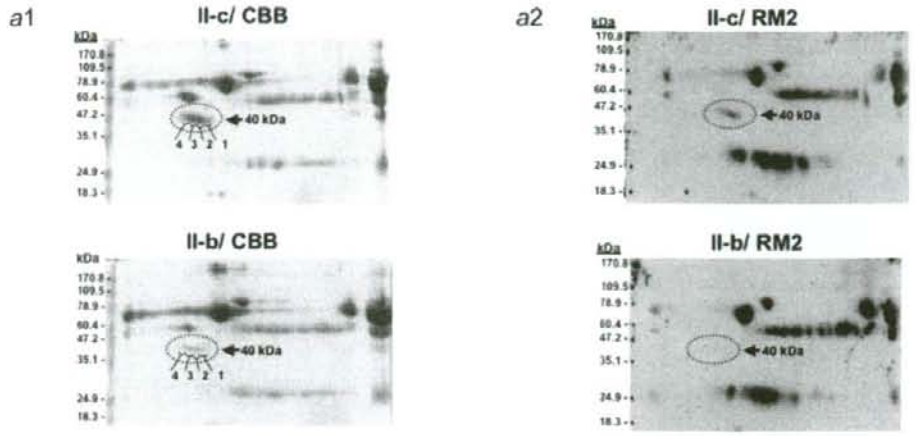
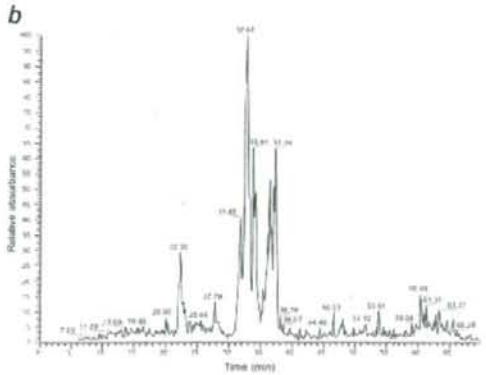


FIGURE 2 – Changes of RM2 reactivity to GPX after radical prostatectomy. (Panel *a*) Examples of RM2 reactivity to sera before and after radical prostatectomy. Preoperative PSA level in each case was indicated at bottom. In all five cases radical prostatectomy Gleason scores were 7 and pT were 2b but No. 10 (3a). b, Before radical prostatectomy; a, after radical prostatectomy; M, size marker. (Panel *b*) Changes of RM2 reactivity to GPX after radical prostatectomy in 15 patients. Longitudinal axis: RM2 reactivity to GPX, RP: radical prostatectomy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]





c

File No.	Sequence
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4	K.DIAPTLTLYVGKK.Q
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6	R.VGYVSGWGR.N
7	K.YVMLPVADQDQC*IR.H
8	K.YVMLPVADQDQC*IR.H
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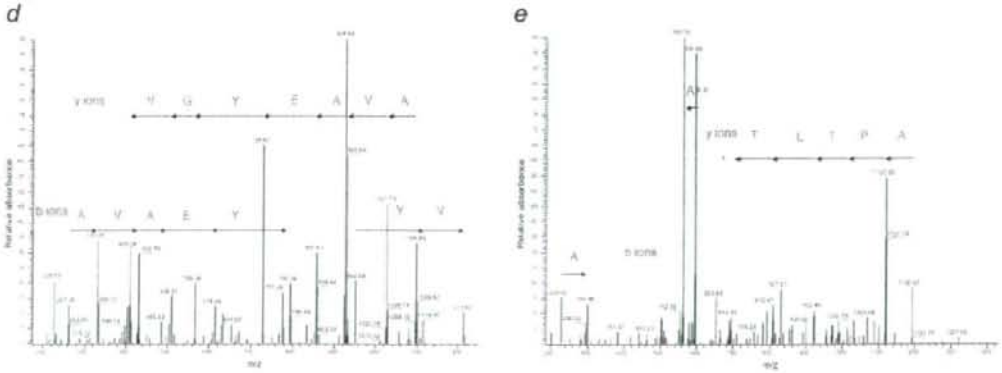

FIGURE 3 – Identification of GPX as haptoglobin-β chain. (Panel *a-1*) Protein components separated by 2D SDS-PAGE: Staining by Coomassie Brilliant Blue (CBB). Agilent column-adsorbed fractions (fraction II) from nonmalignant serum and from prostate cancer serum were analyzed by 2D SDS-PAGE. *Upper*: The pattern from sample II-c (fraction II from serum of malignant subject c). *Lower*: The pattern from sample II-b (fraction II from serum of nonmalignant subject b). Horizontal direction: isoelectric focusing (IEF). Vertical direction: SDS-PAGE. Area circled by dotted line on both left and right: GPX. Note that sample II-c (malignant) has higher CBB staining than sample II-b (nonmalignant). For each sample, GPX is separated into four contiguous spots with different IEF, termed 1, 2, 3 and 4 as indicated. (Panel *a-2*) Upper and Lower. Same as in upper and lower in panel a-1, but stained by mAb RM2. Note that RM2-stained bands are clearly seen for sample II-c but not for sample II-b. (Panel *b*) LC-MS chromatogram of spot 2 on the 2D SDS-PAGE in Panel a after *in gel* trypsin digestion and extraction of the tryptic peptides. Abscissa: LC retention time. Ordinate: MS1 of total ion chromatogram. (Panel *c*) Identification of haptoglobin. (Panel *d*) MS/MS spectrum of m/z 680 at the $[M + 2H]^{2+}$ ion. (Panel *e*) MS/MS spectrum of m/z 710 at the $[M + 2H]^{2+}$ ion. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

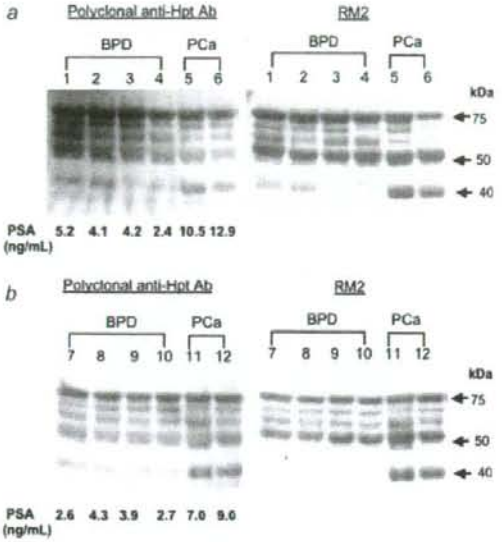

FIGURE 4 – Preferential reactivity of mAb RM2 toward haptoglobin-β chain from prostate cancer. (Panel *a* and *b*) Examples of reactivity to sera of BPD and PCa by polyclonal anti-haptoglobin antibody and mAb RM2, respectively. Left and right panels are from the same patients. *Left panel*: Reactivity to sera by polyclonal anti-haptoglobin antibody. *Right panel*: Reactivity to sera by mAb RM2. PSA level in each case was indicated at bottom. Hpt: haptoglobin, Ab: antibody.

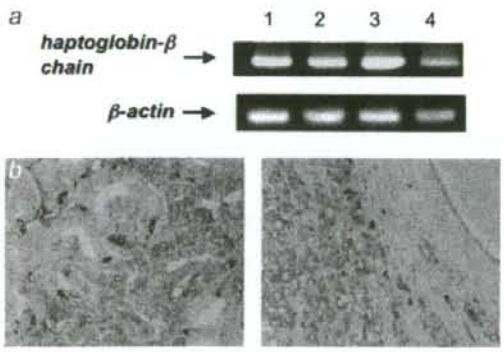

FIGURE 5 – Increased expression of haptoglobin in prostate cancer cells. (Panel *a*) Level of haptoglobin-β mRNA in prostate cancer cell line. 1: LNCaP, 2: PC3, 3: DU145, 4: PrEC. (Panel *b*) Examples of immunohistochemical staining of prostate cancer by polyclonal anti-haptoglobin antibody. Left: Gleason pattern 3, Right: Gleason pattern 4.

blotting by mAb RM2. RM2-blotted spots were strong for prostate cancer specimen II-c but absent for nonmalignant specimen II-b (Fig. 3*a-2*, upper and lower panels). The contiguous spots 1, 2, 3 and 4 as above from specimen II-c were excised from the gel, and subjected to *in gel* digestion by trypsin, followed by LC-MS/MS for protein identification. An example of a base peak chromatogram for tryptic peptides of spot 2 of GPX is shown in Figure 3*b*. The results of the TurboSEQUEST search on the data were displayed in Figure 3*c*, showing human haptoglobin-2 precursors

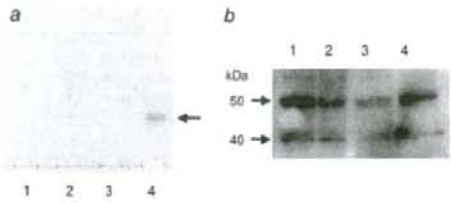

FIGURE 6 – RM2 reactivity is largely due to haptoglobin-β chain in prostate cancer cells. (Panel *a*) TLC immunostaining by mAb RM2. Total glycolipid equivalent to 15 mg of wet weight cell pellet was applied per lane. Lane 1: PC3, lane 2: LNCaP, lane 3: AICaP1, lane 4: TOS1. Arrow: GalNAcDSLc4. (Panel *b*) Comparison of Western blotting of the protein extract of DU145 before and after treatment with the hemoglobin column. The major glycoprotein with approximate molecular mass of 50 kDa is thought to be a form of haptoglobin-α and -β chain complex. Lane 1: protein extract untreated, lane 2: fraction I (the eluate of protein extract passed through the column without incubation), lane 3: fraction II (the eluate of protein extract passed through the column 24 hr after incubation with the column beads), lane 4: wash I (the washed fraction after collecting fraction II).

(P00738). There were 9 tryptic peptides found, which corresponded to 25% coverage of the haptoglobin precursor. However, the protein precursor consists of signal peptide (1–18 residue), α-chain (19–160 residue) and β-chain (162–406 residue).[17–20] All tryptic peptides identified were from β-chain. The corresponding peptide coverage for haptoglobin β-chain (40 kDa) was 38.8% for spot 2. The peptides found for the other 3 spots were similar, also identifying GPX as the haptoglobin β-chain protein. The coverage for spots 1, 3 and 4 was 35.5, 20.0 and 35.5%, respectively. Figure 3*d* shows the MS/MS spectrum of a doubly charged precursor ion at *m/z* 680 identifying SC(PAM)AVAEYGVYVK peptide with annotated amino acid sequence. Figure 3*e* shows the MS/MS spectrum of $[M + 2H]^{2+}$ at *m/z* 710 of peptide DIAPTLTLYVGKK. The amino acid sequences in Figures 3*d* and 3*e* correspond to the residues 380–391 and 216–228 of the haptoglobin precursor.

Preferential reactivity of mAb RM2 toward haptoglobin-β chain from prostate cancer when compared with polyclonal anti-haptoglobin antibody

The profile of reactivity to serum with mAb RM2 was very similar to that with polyclonal anti-haptoglobin antibody (Figs. 4*a* and 4*b*). This finding may support the results of the proteomics analysis described earlier, and also suggests that the 4 bands above haptoglobin-β chain may be various forms of haptoglobin-α and -β chain complex because haptoglobin occurs *in vivo* as polymers of an α and β chain complex.[21] In addition, RM2 showed preferential reactivity toward haptoglobin-β chain from prostate cancer rather than that from BPD when compared with polyclonal anti-haptoglobin antibody (Figs. 4*a* and 4*b*).

Increased expression of haptoglobin in prostate cancer cells

Increased level of haptoglobin-β mRNA was observed in prostate cancer cell lines, LNCaP, PC3 and DU145, when compared with PrEC, normal human prostate epithelial cells (Fig. 5*a*). In immunohistochemical staining, negative to weak staining of polyclonal anti-haptoglobin antibody was observed in benign prostatic glands or stroma. Higher expression of polyclonal anti-haptoglobin antibody in prostate cancer cells was observed in 9 of 20 cases (Fig. 5*b*), whereas expression level was not clearly different between cancer cells and benign glands or stroma in 11 cases. There was no significant association between Gleason score and expression level of polyclonal anti-haptoglobin antibody in prostate cancer cells (data not shown).

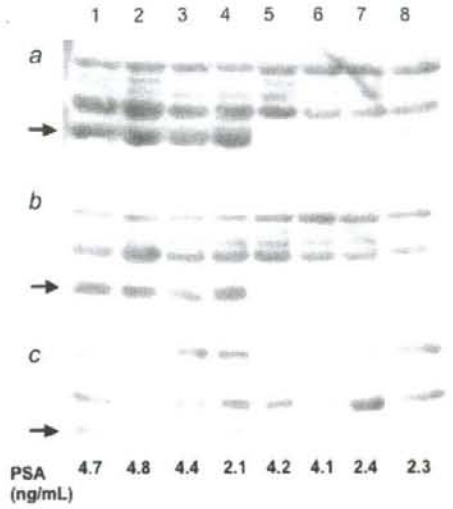

FIGURE 7 – Involvement of glycosylation in RM2 reactivity to serum haptoglobin-β chain. (Panel *a*) No treatment, (Panel *b*) treatment with β-hexosaminidase and (Panel c) treatment with β-hexosaminidase followed by NDV sialidase. Arrow: haptoglobin-β chain. 1–4: sera from prostate cancer patients, 5–8: sera from BPD patients. PSA level for each case was indicated at bottom.

RM2 reactivity is largely due to haptoglobin-β chain in prostate cancer cells

Thin-layer chromatography immunostaining of glycolipids by mAb RM2 showed that expression of GalNAcDSLc4 was not observed in prostate cancer cell lines, whereas it was detected in RCC cell line TOS1 as a positive control (Fig. 6*a*). Expression of GalNAcDSLc4 was neither detected in culture media of prostate cancer cells nor in that of TOS1 cells, whereas MSGb5 as a positive control was detected in supernatant of ACHN cells (data not shown). Most of RM2 reactivity disappeared after treatment with the hemoglobin column (Fig. 6*b*, lane 3). These results indicate that RM2 reactivity in prostate cancer cells is largely due to haptoglobin-β chain.

Involvement of glycosylation in RM2 reactivity to serum haptoglobin-β chain

RM2 reactivity to serum haptoglobin-β chain was little changed after treatment with β-hexosaminidase (Fig. 7*b*) or sialidase from NDV (data not shown). However, most of RM2 reactivity to serum haptoglobin-β chain disappeared after treatment with β-hexosaminidase followed by NDV sialidase (Fig. 7*c*).

Discussion

In the current study, we found that haptoglobin-β chain defined by RM2 is a novel marker, which significantly increased in sera of early prostate cancer. Furthermore, RM2 showed preferential reactivity toward haptoglobin-β chain derived from sera of prostate cancer, suggesting haptoglobin-β chain is qualitatively different between prostate cancer and BPD, and haptoglobin-β chain defined by RM2 is more associated with prostate cancer than BPD. Qualitative change of haptoglobin was also suggested in head and neck cancer since haptoglobin derived from cancer sera was immunosuppressive whereas that from normal sera was not.[22] Thus, both quantitative and qualitative changes of haptoglobin-β chain may be the basis of significant difference of RM2 reactivity to haptoglobin-β chain between prostate cancer and BPD. We also examined whether GalNAcDSLc4 as a ganglioside recognized by mAb RM2 was observed in prostate cancer cells. However, GalNAcDSLc4 was not detected in cell pellets or culture supernatants of prostate cancer cell lines. Furthermore, most of RM2 reactivity to protein extract from prostate cancer cells disappeared after incubation with the hemoglobin column. These results indicate RM2 reactivity to prostate cancer cells largely depends on haptoglobin-β chain.

Because haptoglobin-β chain defined by RM2 also increased in the other urogenital cancers, *i.e.*, it is not specific to prostate cancer, it may be useful for detection of early prostate cancer when coupled with PSA as organ-specific marker. For clinical application, men with lower level of haptoglobin-β chain defined by RM2 may be a problem. Although the number of cases examined was small, transition zone cancer predicted lower level of RM2 reactivity when compared with peripheral zone cancer. Considering that transition zone cancer demonstrates more favorable pathologic features,[23] the method that among men with increased PSA (4–10 ng/mL), those with increased level of haptoglobin-β chain defined by RM2 undergo prostate biopsy, and those without increase in haptoglobin-β chain undergo periodical PSA check and have prostate biopsy when rising PSA is observed may be one of the options for cancer screening. However, a large number of cases are necessary to confirm the relationship between haptoglobin-β chain defined by RM2 and the origin of index cancer.

mAb RM2 was originally established toward disialoganglioside and later found to recognize the glycosyl epitope (β1.4-GalNAcDSLc4).[7,8] In the present study, the proteomics approach showed that mAb RM2 also reacted with haptoglobin-β chain. Based on the findings of the current study, glycosylation status of serum haptoglobin-β chain was examined.[16] Fujimura found that haptoglobin-β chain has minor O-glycosylation site in addition to 4 *N*-glycosylation sites,[19,20] and glycosylation status of serum haptoglobin-β chain was different between prostate cancer and BPD.[16] However, the glycosyl epitope (β1,4-GalNAcDSLc4) recognized by mAb RM2 was not detected on haptoglobin-β chain.[16] Nevertheless, large amount of serum is necessary for thorough analysis of glycosylation to conclude whether the RM2 glycosyl epitope exists or not on haptoglobin-β chain (personal communication with Dr. Fujimura). In the current study, RM2 reactivity to haptoglobin-β chain was little changed after treatment with β-hexosaminidase or NDV sialidase, but most of it disappeared after treatment with β-hexosaminidase followed by NDV sialidase. Because mAb RM2 showed reactivity to DSLc4 in addition to GalNAcDSLc4,[8] changes of RM2 reactivity to haptoglobin-β chain after β-hexosaminidase/sialidase treatment may be compatible with RM2 reactivity to the glycosyl epitope (β1.4-GalNAcDSLc4). However, the existence of RM2 glycosyl epitope has not been verified on haptoglobin-β chain. Therefore, these results only indicate that glycosylation is involved in RM2 reactivity to haptoglobin-β chain, although it remains to be answered whether RM2 reacts directly with a glycosyl epitope or with possible conformational changes induced by glycosylation.

As to the production site, haptoglobin also known as an acute-phase protein has been reported to be mainly produced by liver and secreted into the bloodstream.[24] In the patients with cancer, haptoglobin could be either produced by the tumor cells[25] or the normal cells in response to the stimuli such as IL-6 produced from the tumor cells.[26] Increased expression of haptoglobin in prostate cancer cells as suggested by RT-PCR and immunohistochemistry in the current study indicates that haptoglobin could be produced by cancer cells. In addition to quantitative increase, qualitative change of molecule also indicates haptoglobin is produced from cancer cells since modification of proteins such as aberrant glycosylation could be induced with carcinogenesis. Thus, the previous and the current studies indicate that haptoglobin could be produced from prostate cancer cells. Because haptoglobin occurs *in vivo* as polymers of an α and β chain complex,[21] elevation of

haptoglobin-β chain may be explained by dissociation of α and β chain from haptoglobin.[27] However, although cleavage of haptoglobin by protease is assumed to be responsible for elevation of haptoglobin-α and -β chains, the exact mechanism of elevation of these chains remains to be clarified.[27]

There has been accumulating evidence of haptoglobin expression in sera of various cancers. Haptoglobin-α or -β chain is upregulated in sera of ovarian cancer, breast cancer, acute myeloid leukemia, hepatocellular carcinoma, small cell lung cancer and RCC.[27–32] Furthermore, haptoglobin-β chain carrying α1 Fuc residue, blotted by *Aleuria aurantia* lectin, is significantly enhanced in sera of gastric cancer, colon cancer, hepatocellular carcinoma and pancreatic cancer.[25] In the present study, increase in haptoglobin-β chain defined by RM2 was observed in sera of RCC, urothelial carcinoma and testicular germ cell tumors as well as prostate cancer. Because of this universal increase in haptoglobin-α or -β chain in various malignancies, haptoglobin-β chain defined by RM2 may have potential of exploiting a new approach to serum diagnosis of cancer other than the urogenital malignancies.

In conclusion, haptoglobin-β chain defined by RM2 is a novel serum marker that may be useful for detection of early prostate cancer when coupled with PSA as organ-specific marker because it is not specific to prostate cancer. However, larger trials are necessary to confirm the findings in the current study. It is also important to examine whether haptoglobin-β chain defined by RM2 can be detected in sera of various malignancies other than the urogenital cancers.

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