Expression of S100A2 and S100A4 Predicts for Disease Progression and Patient Survival in Bladder Cancer

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OBJECTIVES To determine the expression patterns and prognostic value of S100A2 and S100A4 in surgical specimens from radical cystectomy for transitional cell carcinoma of the urinary bladder.

METHODS Immunohistochemical staining for S100A2 and S100A4 was performed in 92 archived radical cystectomy and 38 normal specimens. The immunoreactivity of these proteins was stratified on a 0 to 6 scale and then correlated with the pathologic features and clinical outcome.

RESULTS S100A2 expression was significantly decreased in the bladder cancer specimens compared with the controls ($P < 0.0001$), and S100A4 expression was significantly greater in the bladder cancer specimens ($P = 0.03$). The loss of expression of S100A2 and increased expression of S100A4 were associated with muscle invasion ($P < 0.05$). These alterations in expression were also associated with a greater risk of disease progression and a decreased chance of cancer-specific survival at a median follow-up of 25.3 months ($P < 0.0001$ for both). After adjusting for the effects of the pathologic findings, S100A4 expression remained a significant predictor of disease progression ($P < 0.0001$) and cancer-specific survival ($P < 0.0001$).

CONCLUSIONS S100A4 appeared to be an independent predictor for the treatment outcome in bladder cancer. The expression patterns of S100A2 and S100A4 correlated well with the pathologic stage, disease progression, and cancer-specific mortality. This finding could aid in identifying more biologically aggressive cancers and thus patients who might benefit from more intensive adjuvant therapy. UROLOGY 70: 602–607, 2007. © 2007 Elsevier Inc.

Although pathologic findings are the most common primary prognostic factors, remarkable differences in biologic behavior within each stage and grade category have led to an extensive search for more reliable and powerful molecular markers in bladder cancer. S100 proteins have been shown to be involved in a variety of intracellular and extracellular functions, including cell growth, cell-to-cell communication, energy metabolism, and intracellular signal transduction.[1,2] S100A2 is a tumor-suppressor gene that is typically downregulated in cells acquiring a tumorigenic phenotype, suggesting that S100A2 has an important role in inhibiting cancer progression.[3] Differential expression of S100A2 has been reported in a variety of cancers.[4–6]

S100A4, another member of the S100 protein family, has been shown to be associated with invasion and metastases of malignant tumors[7,8] and to be upregulated in transformed cells and in a variety of cancers, including bladder cancer.[9–19] Davies *et al.*[17] reported overexpression of S100A4 in superficial lesions, which was associated with increased metastatic potential and a greater likelihood of a fatal outcome. Agerbaek *et al.*,[19] using multivariate analyses of data from patients with bladder cancer who had undergone radical cystectomy with preoperative external beam radiotherapy, demonstrated that a high level of S100A4 was a significant predictor of distant metastases and reduced metastasis-free survival.

We hypothesized that S100A2 and S100A4 are biologic markers with prognostic value for bladder cancer. The primary objectives of the present study were to determine the expression patterns of S100A2 and S100A4 and to assess their prognostic value in patients with transitional cell carcinoma of the urinary bladder who had undergone radical cystectomy.

MATERIAL AND METHODS

Patient Population

From March 1990 to October 2004, 118 consecutive patients diagnosed with bladder cancer underwent radical cystectomy

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with pelvic and iliac lymphadenectomy at the Kitasato University Hospital. Of the 118 patients, 26 were excluded from additional analysis because they had undergone preoperative neoadjuvant therapy (either radiotherapy or chemotherapy; n = 19) or had histologic findings other than transitional cell carcinoma (n = 7). No patients had distant metastases at cystectomy. Of the 92 patients analyzed, 75 were men (81.5%) and 17 were women (18.5%). The median patient age was 63 years (range 40 to 81, mean 62.3).

The indications for cystectomy in patients with initial Stage Ta, T1, or Tis bladder cancer included failure of intravesicular therapy (n = 19), multiple recurrences (n = 7), aggressive histopathologic features, including high-grade tumor, presence of carcinoma in situ, or multifocal extensive disease (n = 7), or progression to muscle-invasive cancer (n = 18). The remaining 41 patients underwent cystectomy for an initial presentation of muscle-invasive disease.

The histopathologic characteristics were confirmed by blinded review of the original pathology slides by a single pathologist (K.I.). The 2002 TNM classification was used for pathologic staging, and the World Health Organization classification was used for pathologic grading.[20] Lymphovascular invasion was determined according to the presence of cancer cells within an endothelium space. Cancer cells that had merely invaded a vascular lumen were considered negative.[21] Formalin-fixed, paraffin-embedded blocks representing the most invasive areas of each tumor were collected for additional investigation. The median follow-up was 25.3 months (range 1.1 to 196.1, mean 47.0) for patients alive at the last follow-up visit. When patients died, the cause of death was determined by the treating physicians, chart review corroborated by death certificates, or death certificates alone. Normal bladder urothelium specimens from 38 patients who underwent radical cystectomy without any pathologic findings suggestive of malignancy served as the controls. The ethics committee of Kitasato University Hospital approved the study.

Immunohistochemistry and Scoring

Antigen retrieval was performed by immersing the tissue sections in 0.01 M citrate buffer (pH 6.0) and microwaving for 15 minutes followed by cooling to room temperature. The tissue was then incubated with primary polyclonal antibody against S100A2 (Dako Cytomation, Denmark) at 1:50 concentration and against S100A4 (Dako Cytomation) at 1:25 concentration in a humidified chamber at 4°C for 16 hours. Secondary antibody was applied with the use of Envision (Rabbit, Dako, Denmark). Staining of inflammatory stromal cells for S100A4 and normal urothelium for S100A2 served as positive controls for these staining reactions.[19] Batches in which primary antibodies had been omitted were used as negative controls. All slides were reviewed by a single pathologist (K.I.) who was unaware of the clinical and pathologic data. Each image was interpreted for immunoreactivity by using a semiquantitative 0 to 3+ system that incorporated both staining intensity and the percentage of positive cells (labeling frequency). The intensity grading scale ranged from no detectable signal (0) to a strong signal seen at low-power magnification (3); a specimen's intensity value was that of the highest intensity "hot spot." The labeling frequency was scored as 0 (0%), 1 (1% to 33%), 2 (34% to 66%), or 3 (67% to 100%), with the analysis done using the average of three measurements. The sum index was obtained by totaling the staining intensity and percentage scores.

In a preliminary study, we assessed the discriminative value for bladder cancer characteristics and prognosis of using each value in the sum index as the cutoff point. Kaplan-Meier analyses revealed that a cutoff sum index value of 1 provided the best positive and negative prediction for bladder cancer progression and survival (data not shown). Likewise, the evaluation of the association of each cutoff value for S100A2 and S100A4 with clinical, pathologic, and molecular characteristics showed that 1 was the only value to yield statistically significant findings (data not shown). The expression scores for these proteins were stratified further into normal (score 2 or greater in S100A2 and 0 to 1 in S100A4) and abnormal (score 0 to 1 in S100A2 and 2 or greater in S100A4) for the purposes of the presentation.

Postoperative Follow-up

Each patient was scheduled to have a postoperative follow-up examination every 4 months for the first year, semiannually in the second year, and annually thereafter. More frequent examinations were scheduled if clinically indicated. Of the 92 patients, 17 (18.5%) received adjuvant chemotherapy (methotrexate, vinblastine, doxorubicin, and cisplatin) after surgery for adverse pathologic characteristics, including regional or distant lymph node metastases or extravesical involvement, and 32 (34.8%) received methotrexate, vinblastine, doxorubicin, and cisplatin for disease recurrence.

Statistical Analyses

For the purpose of analysis, the tumor pathologic stage (pT1 or less versus pT2 or greater), grade (grade 1-2 versus grade 3), and lymph node status (N0 versus N1 and N2) were evaluated as dichotomized variables. Fisher's exact test was used to evaluate the association among sex, pathologic stage, pathologic grade, presence of carcinoma in situ, lymph node status, and lymphovascular invasion. The difference in age between the S100A2 and S100A4-positive and negative groups was tested using the Mann-Whitney U test. The Kaplan-Meier method was used to calculate the survival functions, and any differences were assessed with the log-rank statistic. Multivariate survival analyses were performed with the Cox proportional hazards regression model, controlling for S100A2 and S100A4 expression, pathologic stage and grade, and the presence of lymphovascular invasion and lymph node metastases. Statistical significance in this study was set as $P < 0.05$. All reported P values are two-sided. All analyses were performed using the Statistical Package for Social Sciences, version 11.0, for Windows (SPSS, Chicago, Ill).

RESULTS

Association of S100A2 and S100A4 Expression with Clinicopathologic Characteristics

A representative case of immunostaining for S100A2 and S100A4 in normal and cancerous bladder tissue is shown in Figure 1. The normal bladder epithelium had distinct cytoplasmic S100A2 immunoreactivity (Fig. 1A). In contrast, S100A4 staining was scant (Fig. 1B). The expression of S100A4 and S100A2 in bladder cancer was significantly greater ($P = 0.03$) and lower ($P < 0.0001$), respectively, than in normal bladder urothelium (Fig. 1C,D).

Abnormal expression of S100A2 and S100A4 was noted in 57 (62.0%) and 45 (48.9%) of the 92 cancer

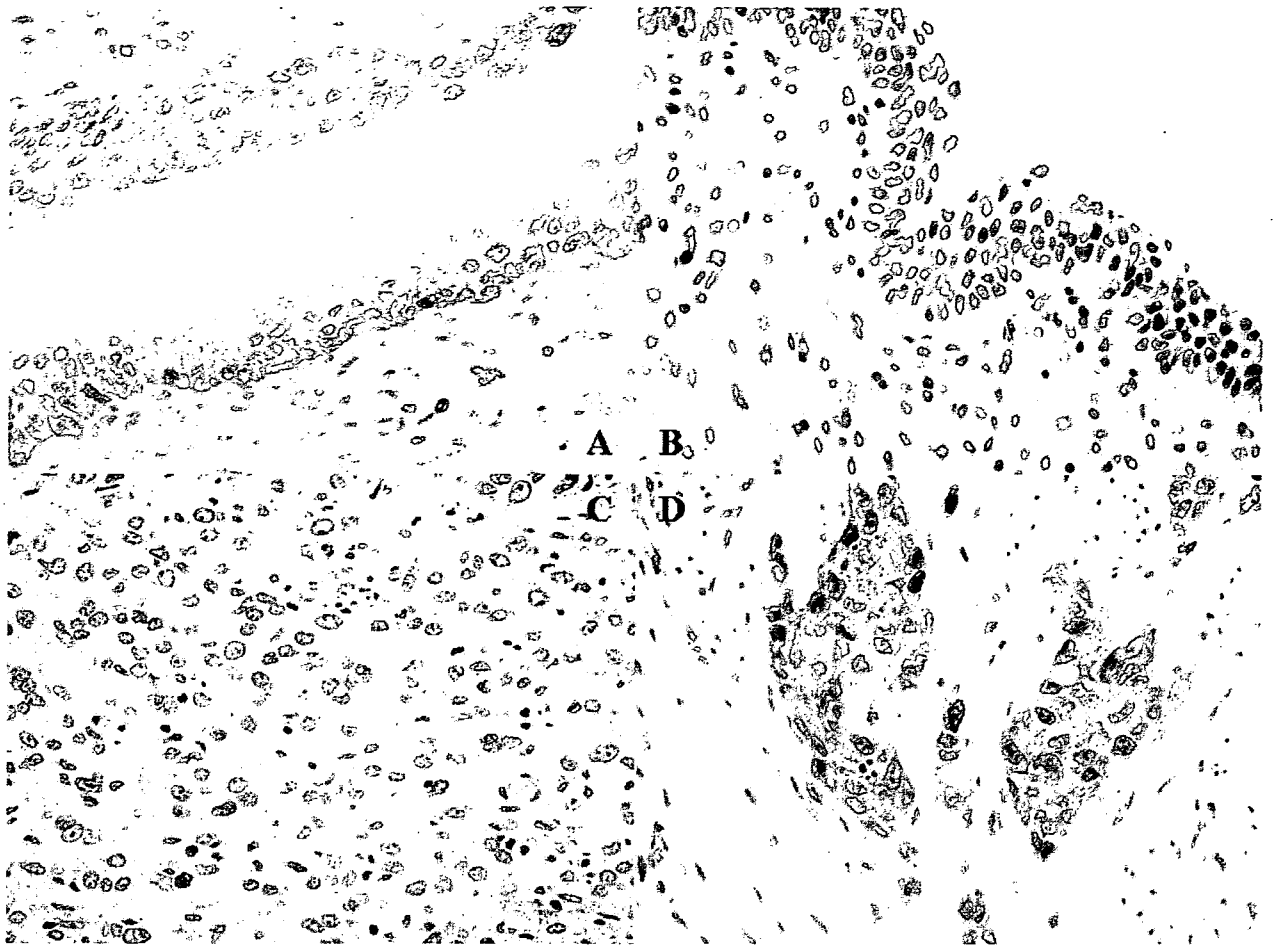

Figure 1. Representative S100A2 and S100A4 immunohistochemical staining in bladder cancer specimens at ×400. **(A)** positive staining of S100A2 in normal urothelium. **(B)** Negative staining of S100A4 in normal urothelium. **(C)** Negative staining of S100A2 in Stage pT2, grade 3 tumor. **(D)** Positive staining of S100A4 in Stage pT4, grade 3 tumor.

specimens. Of the 92 patients, 23 (25%) had tumors with normal expression of S100A2 and S100A4, 24 (26.1%) had abnormal S100A2 and normal S100A4 expression, 12 (13%) had normal S100A2 and abnormal S100A4 expression, and 33 (35.9%) had both abnormal S100A2 and S100A4 expression. The clinicopathologic characteristics of the 92 patients and the association with S100A2 and S100A4 expression are given in Table 1. Abnormal expression of S100A2 and S100A4 was associated with muscle invasion and more advanced disease ($P = 0.03$ and $P = 0.009$, respectively). Age at cystectomy, gender, pathologic grade, presence of carcinoma in situ, lymph node metastases, and lymphovascular invasion did not differ significantly between phenotypes with normal and abnormal expression of either S100A2 or S100A4. A significant inverse relationship was noted between the expression of S100A2 and S100A4 ($P = 0.03$).

Association of S100A2 and S100A4 Expression with Clinical Outcomes

Distant metastases developed in 44 patients (47.8%) at a median of 13.0 months (range 0.6 to 123.3). Of the 92 patients, 40 (43.5%) were dead at the analysis (median time to death 19.8 months, range 2.2 to 141.5). The cause of death in 36 patients (90.0%) was identified as advanced metastatic bladder cancer and 4 patients had died of intercurrent causes without evidence of disease progression.

At a median follow-up of 25.3 months, a Kaplan-Meier projection indicated that abnormal expression of S100A2 was not associated with an increased probability of disease progression and bladder cancer-specific death ($P > 0.05$ for both). However, bladder cancer with abnormal S100A4 expression was associated with a significantly increased risk of disease progression and cancer-specific mortality ($P < 0.0001$), and abnormal expression of both S100A2 and S100A4 was associated with the greatest risk of disease progression and cancer-specific death ($P < 0.0001$; Fig. 2). In contrast, patients with tumor with normal expression of both S100A2 and S100A4 had significantly better prognosis.

In Cox proportional hazards regression analyses, S100A4 expression, pathologic grade, lymphovascular invasion status, and lymph node metastases were associated with bladder cancer progression ($P < 0.05$), and S100A4

Table 1. Association of S100A2 and S100A4 expression with clinical and pathologic characteristics of patients who underwent radical cystectomy for bladder TCC

Characteristic	Patients (n)	S100A2 Expression		P Value*	S100A4 Expression		P Value*
		Normal	Abnormal		Normal	Abnormal	
Total	92	35 (38.0)	57 (62.0)		47 (51.1)	45 (48.9)	
Gender				0.58			0.99
Male	75 (81.5)	30 (40.0)	45 (60.0)		38 (50.7)	37 (49.3)	
Female	17 (18.5)	5 (29.4)	12 (70.6)		9 (52.9)	8 (47.1)	
Pathologic stage				0.03			0.009
Pa, Pis, P1	24 (26.1)	14 (58.3)	10 (41.7)		18 (75.0)	6 (25.0)	
P2–P4	68 (73.9)	22 (32.4)	46 (67.6)		29 (42.6)	39 (57.4)	
Pathologic grade				0.99			0.09
Grade 1–2	46 (50.0)	18 (39.1)	28 (60.9)		28 (60.9)	18 (39.1)	
Grade 3	46 (50.0)	17 (37.0)	29 (63.0)		19 (41.3)	27 (58.7)	
Carcinoma in situ				0.08			0.58
Negative	77 (83.7)	26 (33.8)	51 (66.2)		38 (49.4)	39 (50.6)	
Positive	15 (16.3)	9 (60.0)	6 (40.0)		9 (60.0)	6 (40.0)	
Lymph node status†				0.99			0.31
N0	66 (71.7)	26 (39.4)	40 (60.0)		37 (56.1)	29 (43.9)	
N1–N2	20 (21.7)	8 (40.0)	12 (60.0)		8 (40.0)	12 (60.0)	
Lymphovascular invasion‡				0.63			0.11
Negative	33 (35.9)	12 (36.4)	21 (63.6)		21 (63.6)	12 (36.4)	
Positive	45 (48.9)	14 (31.1)	31 (68.9)		20 (44.4)	25 (55.6)	

TCC = transitional cell carcinoma.
Data presented as number of patients, with percentages in parentheses.
* Fisher's exact test (two sided).
† Lymph node pathologic status of 6 patients was unknown.
‡ Lymphovascular status of 14 patients was unknown.

expression and lymphovascular invasion status were associated with cancer-specific survival after controlling for other prognostic variables ($P < 0.05$). Abnormally expressed S100A4 had the greatest odds ratio for disease progression ($P < 0.0001$, odds ratio 7.01, 95% confidence interval 2.72 to 18.1) and cancer-specific mortality ($P < 0.0001$, odds ratio 8.25, 95% confidence interval 2.84 to 23.9).

COMMENT

S100A4, one of the members of the S100 protein family, plays a role in regulating the cell cycle and cell motility, as well as in modulating intercellular adhesion.[7,8] Upregulation of S100A4, shown in a variety of human carcinomas, including bladder cancer,[9–19] has been associated with disease progression, metastasis, and decreased patient survival.[17,19,22] We also found S100A4 was associated with the pathologic stage in patients who had undergone radical cystectomy. The outcome of those with tumors with abnormally expressed S100A4 was significantly worse than that for those with normal expression. Abnormally expressed S100A4 had the greatest odds ratio of any factor examined for disease progression and cancer-specific mortality, reflecting the biologic aggressiveness of bladder cancers with this phenotype.

Although the association of S100A2 expression with prognosis has been suggested for other malignancies,[4–6] abnormal (ie, reduced) expression of S100A2 in this series of bladder cancer appeared to be less prognostic than the abnormal expression of S100A4. S100A2 expression is regulated during the cell cycle, with levels increasing as cells enter the S phase and induced by growth factors in the early G_1 phase of the normal cell cycle.[3] Because S100A2 is believed to be regulated by the tumor-suppressor p53[23]—induction of p53 activity through cell cycle arrest by DNA damage results in increased S100A2 transcription[24]—the loss of S100A2 expression could contribute to increased resistance to cell death. In addition, overexpression of S100A4 promotes p53 degradation, which could be one mechanism through which S100A4 supports cancer metastasis.[25] The effect of reduced S100A2 expression and overexpressed S100A4, mediated by alterations in p53 level, could be additive, because simultaneous abnormal expression of S100A2 and S100A4 was associated with the direct prognosis.

We found an inverse relationship between the expression of S100A2 and S100A4 protein in bladder cancer. Reduced and increased expression of S100A2 and S100A4, respectively, was associated with enhanced biologic aggressiveness and more advanced pathologic features, as well as reductions in the time to progression and likelihood of patient survival.

Although the mechanism of action of S100A4 is largely unknown, its expression did not result in the spontaneous formation of mammary tumors in a transgenic mouse model.[26] In contrast, overexpression of S100A4 by gene transfection enhanced the ability to form metastatic lesions in animal models of bladder cancer.[27] S100A4 could interact with changes in other key proteins such as p53 to

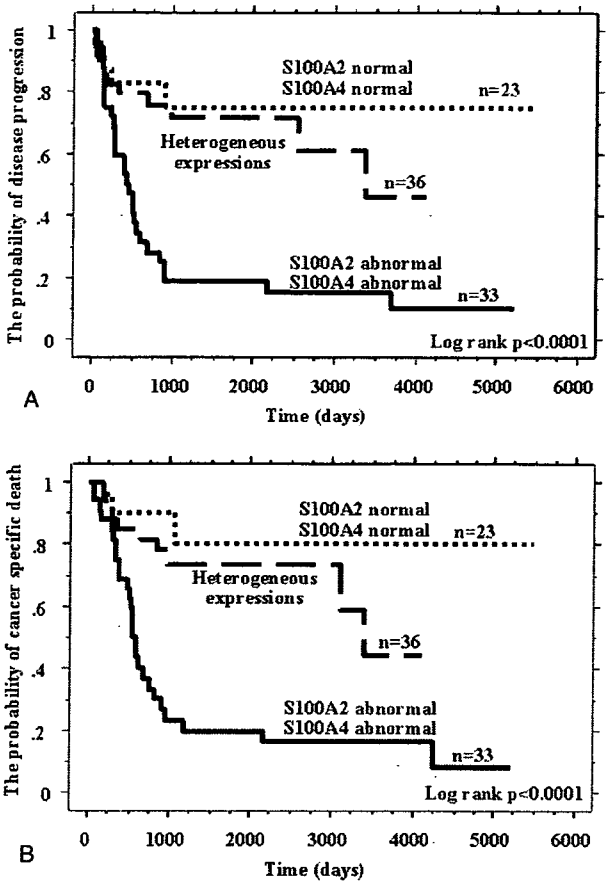

Figure 2. Probability of bladder cancer progression **(A)** and mortality **(B)** after radical cystectomy according to expression of S100A2 and S100A4.

promote tumor aggressiveness, rather than itself playing a principal role in tumor development.

Our study was limited by its retrospective nature and relatively small number of patients. Because a single pathologist assessed the immunohistochemical outcome in a blinded fashion, interobserver variance was not tested in this study. Tumor sampling bias could have affected the outcome to some extent, although three separate tumor sites were selected to reduce this problem. Thus, the reproducibility of our findings should be tested by other investigators.

Although the pathologic findings currently serve as a principal determinant to predict tumor biology, analyses of new protein markers such as S100A2 and S100A4 could identify those who might benefit most from undergoing interventional adjuvant therapy. Additional prospective studies with a larger number of patients are needed to validate our findings.

CONCLUSIONS

S100A4 predicted for disease progression and bladder cancer-specific mortality in those who had undergone radical cystectomy. Abnormal S100A2 and S100A4 expression was associated with a more advanced pathologic stage. Patients with tumor that had increased and reduced expression of S100A2 and S100A4, respectively, had a better prognosis, and those with elevated expression of S100A4 in the absence, or scant expression, of S100A2 were at a substantially increased risk of disease progression and cancer-specific death. The simultaneous analysis of S100A2 and S100A4 expression could aid in identifying more biologically aggressive cancer and patients who might benefit most from interventional therapy.

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Impact of Unilateral Sural Nerve Graft on Recovery of Potency and Continence Following Radical Prostatectomy: 3-Year Longitudinal Study

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Purpose: We conducted a 3-year longitudinal study assessing the impact of unilateral sural nerve graft on recovery of potency and continence following radical prostatectomy.

Materials and Methods: A total of 113 patients undergoing radical retropubic prostatectomy were classified into 3 groups according to the degree of nerve sparing, that is unilateral nerve preservation with contralateral sural nerve graft interposition, bilateral nerve sparing and unilateral nerve sparing. Urinary continence and potency were estimated by the UCLA Prostate Cancer Index questionnaire.

Results: Patients in the nerve sparing plus sural nerve graft group were younger than those in the bilateral nerve sparing or unilateral nerve sparing groups. At baseline the unilateral nerve sparing plus sural nerve graft group and the bilateral nerve sparing group reported better sexual function than the unilateral nerve sparing group (62.1 and 61.5 vs 49.9, $p < 0.05$). The bilateral nerve sparing group showed more rapid recovery than the unilateral nerve sparing plus sural nerve graft group after radical retropubic prostatectomy ($p < 0.01$). After 24 months there were no significant differences observed between the bilateral nerve sparing and the unilateral nerve sparing plus sural nerve graft group (28.7 vs 32.9). The bilateral nerve sparing group reported a better sexual function score than the unilateral nerve sparing group throughout the postoperative period ($p < 0.05$). The bilateral nerve sparing group maintained significantly better urinary function at 1 month after radical retropubic prostatectomy than the unilateral nerve sparing plus sural nerve graft group ($p < 0.05$). After 3 months these groups were almost continent. The unilateral nerve sparing group reported lower urinary function scores during the first year compared to the other groups.

Conclusions: The nerve graft procedure may contribute to the recovery of urinary function as well as sexual function after radical retropubic prostatectomy. This finding needs to be validated in a randomized trial.

Key Words: prostatic neoplasms, prostatectomy, sural nerve, urinary incontinence, impotence

Prostate cancer has a significant impact on HRQOL. Although a variety of treatment options are available including external beam radiation, brachytherapy and hormonal ablation, radical prostatectomy is considered a safe and effective treatment for localized prostate cancer.[1] Urinary incontinence and erectile dysfunction represent the principal sources of postoperative adverse events for patients who have undergone RP. Because initiation of penile erection is a neurovascular event, preservation of the cavernous nerves during RP is the most important factor for the recovery of erectile function following RP. Catalona et al reported excellent results with overall postoperative potency rates of 68% and postoperative continence rates of 92%.[2] With low volume and low stage disease nerve sparing does not compromise surgical margins. However, nerve sparing might not be appropriate in men with high grade tumors or palpable disease extending toward the neurovascular bundle. Interposition of sural nerve graft to replace resected cavernous nerves during RP confers a greater chance of recovering erectile function than without grafts. Scardino and Kim reported that with nerve grafting for the side of NVB resection, erectile function of the patients undergoing unilateral nerve sparing returns to a level approximating bilateral nerve sparing.[3] On the other hand, several studies have shown that preservation of the NVB is also associated with improved recovery of urinary control after RP.[4,5]

Although several investigators have reported short-term results with nerve grafting, there is still controversy regarding the long-term outcomes of nerve grafts following RP. We report longer term patterns of HRQOL (ie potency and continence) recovery during the first 3 years after RP using a validated questionnaire.

PATIENTS AND METHODS

Patient Population and Operative Technique

From January 2002 to December 2004 a total of 145 patients with newly diagnosed localized prostate cancer were treated with RP at Tohoku University Hospital. There were 15 patients with nonnerve sparing and 3 with bilateral sural

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nerve graft excluded from analysis. An additional 14 patients were excluded who received initial hormonal ablation, leaving 113 candidates for this study. These patients were classified into 3 groups according to the degree of nerve sparing, that is unilateral nerve preservation with contralateral sural nerve graft interposition group (UNS plus SNG), a bilateral nerve sparing group and a unilateral nerve sparing group. The indications for nerve sparing procedure depended on preoperative factors (clinical stage, transrectal ultrasound findings, number and Gleason score of positive biopsies, PSA or patient preference) and intraoperative factors, prioritizing cancer control. All patients who had minimal erectile dysfunction and in whom nerve resection was anticipated were offered SNG and counseling regarding the risks, benefit and likely impact on postoperative potency recovery. Patients ultimately decided whether SNG interposition would be performed. In our study preservation of the NVB was assigned based on the results of intraoperative electrostimulation as reported by Kurokawa et al.[6]

Quality of Life Assessment

Urinary continence and potency were estimated using the urinary and sexual function and bother domains of the UCLA PCI, which assesses prostate specific HRQOL.[7] The questionnaire had already been translated into Japanese, and the validity and reliability had been previously tested.[8] All patients were informed of their cancer diagnosis before being asked to fill out the questionnaires. Followup interviews were conducted in person at scheduled study visits of 1, 3, 6, 12, 18, 24 and 36 months after RP. All patients who agreed to participate in this study received a questionnaire, an informed consent form and a prepaid postage envelope for returning the questionnaire. They voluntarily provided the self-reported questionnaire by mail.

Statistical Analyses

At baseline a comparison among the 3 groups was performed using the chi-square test or 1-way analyses of variance (ANOVA). UCLA PCI scores for the various domains are shown as the mean plus or minus standard deviation (SD) on 0 to 100 scales, with higher scores always representing better outcomes. Statistical analyses were performed using repeated ANOVA or the Mann-Whitney U test for groups to compare the effects of each treatment, with $p < 0.05$ considered statistically significant.

RESULTS

Complete demographic and clinical data were available for participants at enrollment. Table 1 compares these data among 3 groups. The age of the UNS plus SNG group was statistically lower than that of the BNS or UNS groups ($p < 0.05$ for each). The 3 groups were comparable in terms of preoperative PSA, Gleason scores and pathological tumor stage. Each group showed similar levels of comorbidities and sociodemographic characteristics. Some patients (50.4%) experienced comorbidities, the most common of which were hypertension (26%), diabetes (7%), gastrointestinal (18%), cardiovascular (9%) disease and other kinds of carcinoma (5%), but these comorbidities have been well controlled. There were 6 patients (5%) who received salvage therapy because of biochemical recurrence. All patients received bicalutamide or radiotherapy. No patients used vacuum erection devices.

TABLE 1. *Demographic and clinical characteristics of study population*

	UNS + SNG	BNS	UNS	p Value
No. pts	19	34	60	
Age at survey:				
Mean ± SD	58.0 ± 5.4	64.1 ± 5.8	65.1 ± 5.7	<0.001*
Median	58	64	65	
Range	48–69	47–73	51–77	
PSA at diagnosis (ng/ml):				
Mean ± SD	8.0 ± 4.7	8.3 ± 8.9	8.8 ± 6.7	0.878†
Median	6.5	6.4	7.3	
Range	3.4–21.8	3.1–53.0	2.1–52.7	
No. clinical tumor stage:				0.046†
T1	14	30	39	
T2	3	4	19	
T3	2	0	2	
No. pathological tumor stage:				0.183†
T2	16	32	48	
T3	3	2	12	
No. Gleason score:				0.857†
6 or Less	8	14	28	
7 or Greater	11	20	32	
No. salvage therapy ablation (%)	1 (5)	2 (5)	3 (5)	0.577†
No. comorbidities:				0.607†
None	12	18	27	
1–2	6	13	29	
3+	1	3	4	
No. working status:				0.461†
Full-time	10	11	29	
Part-time	4	5	6	
Retired/no job	5	14	25	
No. marital or relationship status:				0.842†
Married or living with spouse or partner	17	31	56	
Unmarried or not in significant relationship	2	3	4	

* Mann-Whitney U test.
† Chi-square test.

TABLE 2. *UCLA PCI scores*

	Mean ± SD			
	UNS + SNG	BNS	UNS	p Value
Urinary function:				<0.001
Baseline	95.9 ± 11.9	95.5 ± 12.8	97.4 ± 6.8	
1 Mo	51.5 ± 32.1*	66.8 ± 23.9*	56.4 ± 26.1*	
3 Mos	73.4 ± 27.6*	71.4 ± 21.1*	61.2 ± 25.4*	
6 Mos	82.3 ± 24.8*	84.2 ± 16.9*	75.8 ± 30.1*	
12 Mos	84.0 ± 22.1*	84.2 ± 15.3*	76.2 ± 24.3*	
18 Mos	88.8 ± 14.3*	87.0 ± 14.6*	81.3 ± 19.9*	
24 Mos	89.0 ± 12.9	88.7 ± 14.2	86.4 ± 18.9*	
36 Mos	90.6 ± 6.5	88.5 ± 20.6	86.3 ± 13.6*	
Urinary bother:				<0.001
Baseline	93.3 ± 11.6	91.1 ± 13.6	94.5 ± 8.9	
1 Mo	58.3 ± 38.6*	74.0 ± 25.0*	65.5 ± 28.6*	
3 Mos	81.9 ± 24.7*	79.6 ± 25.4*	70.3 ± 34.4*	
6 Mos	83.8 ± 20.9*	83.3 ± 28.4*	77.4 ± 29.8*	
12 Mos	86.8 ± 22.0	86.4 ± 14.6	82.1 ± 24.0*	
18 Mos	91.7 ± 10.5	89.6 ± 12.3	85.3 ± 20.5	
24 Mos	93.3 ± 9.0	89.4 ± 9.3	83.0 ± 16.6	
36 Mos	91.7 ± 7.9	88.5 ± 12.2	87.5 ± 16.5	
Sexual function:				<0.001
Baseline	62.1 ± 11.6	61.5 ± 16.9	49.9 ± 19.4	
1 Mo	7.2 ± 7.6*	12.7 ± 20.8*	6.5 ± 8.2*	
3 Mos	7.9 ± 4.4*	16.0 ± 18.6*	6.7 ± 8.6*	
6 Mos	11.9 ± 6.8*	22.6 ± 17.2*	8.4 ± 10.1*	
12 Mos	16.7 ± 12.6*	24.4 ± 17.4*	13.2 ± 14.0*	
18 Mos	24.2 ± 12.0*	25.5 ± 21.5*	13.9 ± 12.0*	
24 Mos	27.7 ± 10.4*	26.5 ± 20.9*	12.7 ± 12.9*	
36 Mos	32.9 ± 17.0*	28.7 ± 28.7*	13.4 ± 13.4*	
Sexual bother:				<0.001
Baseline	80.3 ± 21.7	81.9 ± 20.3	78.8 ± 22.7	
1 Mo	31.3 ± 27.2*	45.6 ± 31.1*	48.4 ± 32.4*	
3 Mos	33.3 ± 35.4*	48.9 ± 33.5*	47.8 ± 36.4*	
6 Mos	44.5 ± 30.0*	52.1 ± 28.1*	50.2 ± 34.9*	
12 Mos	39.0 ± 23.4*	59.4 ± 30.5*	48.1 ± 32.2*	
18 Mos	51.7 ± 19.2*	54.4 ± 28.6*	55.0 ± 25.7*	
24 Mos	50.0 ± 19.6*	50.0 ± 20.4*	45.0 ± 29.2*	
36 Mos	58.3 ± 23.6*	45.8 ± 30.0*	54.6 ± 30.6*	

* Statistically significant changes from baseline ($p < 0.05$).

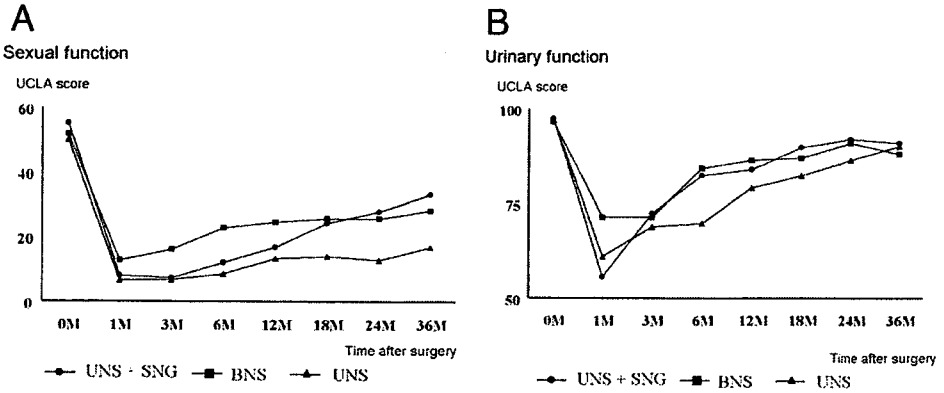
Table 2 presents the recovery of urinary and sexual domains of each group. At baseline the UNS plus SNG and the BNS groups reported better sexual function than the UNS group (62.1 and 61.5 vs 49.9, respectively, $p < 0.05$). The BNS group showed more rapid recovery than the UNS plus SNG group within 12 months ($p < 0.01$, repeated ANOVA). However, after 24 months there were no significant differences between the UNS plus SNG and BNS groups. The UNS plus SNG group continued to show improvement even in the third year. The BNS group had a better sexual function score than the UNS group throughout the postoperative period at 3 years. At 36 months 25% of the UNS plus SNG group and 28% of the BNS group considered the ability to function sexually as fair or good compared with 12% of patients with UNS alone. In addition, 60% of the UNS plus SNG group and 55% of the BNS group could achieve an erection more than half the time vs 18% of patients with UNS alone. All of the surgery groups had substantial impairment in the sexual bother domains throughout the postoperative period (Mann-Whitney U test $p < 0.01$ for each point). The UNS plus SNG group claimed lower sexual bother scores than the other 2 groups at 1 and 3 months postoperatively (both $p < 0.05$).

When considering urinary continence no significant differences were observed in urinary function and bother scores at baseline for each group. The BNS group maintained significantly better urinary function at 1 month after RP than the SNG group (66.8 vs 51.5, $p < 0.05$). After 12 months the BNS and SNG groups were almost continent. The UNS group reported lower urinary function scores during the first year than the other groups. Urinary bother at 1 and 3 months was significantly worse than baseline in all 3 groups. However, at 6 months it returned to the baseline in the UNS plus SNG and BNS groups. The UNS group reported lower urinary function scores during the first year than the other groups.

Because age affects sexual and urinary function, we also performed age matched analyses. Among patients younger than 66 years 18 were in the UNS plus SNG group, 21 in the BNS group and 26 in the UNS group. With age matched analysis there was no difference in sexual function at baseline among the 3 treatment groups. The same tendency that the BNS group had more rapid recovery of urinary and sexual function than the SNG group was observed just after RP. The UNS plus SNG group continued to show improvement, and the differences lost significance in sexual and urinary function by 12 and 3 months, respectively (see figure).

DISCUSSION

Nerve grafting is a surgical technique that has been used for decades. The grafted nerve serves primarily as a channel or scaffold for regenerating axons to reestablish the connection between the severed segments. To our knowledge this is the largest published series of sural nerve grafts performed at a



Sequential changes in average sexual function (*A*) and urinary function (*B*) scores in unilateral nerve sparing with contralateral sural nerve graft group bilateral nerve sparing group and unilateral nerve sparing group for age matched comparison.

single institution with the longest clinical followup. Our study has several important findings. With selective graft replacement of a unilateral nerve resection, sexual function appears to recover to a level approximating that of bilateral nerve sparing and superior to that of unilateral resection without grafts. Furthermore, the present longitudinal study revealed different profiles in terms of recovery of sexual function after RP. The exact recovery time for return of full sexual function after nerve sparing RP is still underestimated, and the majority of patients do not recover erectile function as early as urinary continence. Walsh reported that maximal erectile recovery was not witnessed until a mean period of 18 months after bilateral nerve sparing RP because a number of factors such as thermal damage, ischemic injury and the local inflammatory effects of surgical trauma may impair the cavernous nerves.[9] Whereas sexual function was significantly better in the BNS than the UNS plus SNG group immediately after RP, the latter group continued to show improvement after postoperative year 2 and sexual function reached a level approximating that of bilateral nerve sparing. However, the UNS plus SNG group reported a significantly lower sexual bother score than the other 2 groups, suggesting that those who underwent RP with nerve grafting were potentially more interested or motivated to maintain or resume sexual function postoperatively. Therefore, postoperative erectile dysfunction especially within 12 months was a burden and they reported lower sexual bother scores. Although our study may not be large enough to generalize whether nerve grafting actually helps men who would otherwise not achieve erections sufficient for intercourse, these findings will be helpful in counseling patients when they are weighing a decision about RP.

Unilateral nerve grafting RP is beneficial for the early recovery of postoperative urinary continence. Urinary incontinence is a concern particularly relevant to men undergoing RP because surgery more frequently negatively impacts continence than other treatment modalities, and because patients rate urinary status as one of their greatest concerns regarding HRQOL. In a multivariate analysis Eastham et al showed that unilateral nerve preservation was associated with less postoperative incontinence than bilateral NVB resection.[10] Our study revealed that although the BNS group showed better urinary function than the UNS plus SNG group at 1 month after RP, there were no differences at 3 months and both groups improved at comparable rates after 6 months. The precise mechanism behind the functional relationship between nerve sparing and continence remains elusive, and it is most likely multifactorial. NVB preservation may influence continence not only by maintaining efferent but also afferent innervation. The effect of autonomic innervation on the sphincter mechanism was convincingly shown by intraoperative stimulation of NVBs during RP.[11] Singh et al demonstrated that patients who underwent RP with UNS plus SNG had a greater rate of urinary function recovery relative to patients in whom an SNG interposition was not performed, which was similar to our finding.[12] Other factors that may influence urinary function include the surgical methods of bladder neck reconstruction and anastomosis.[10] The current study minimized the confounding influence of surgical technique on urinary function. All patients in this study had the bladder neck reconstructed and anastomosis performed using the same technique.

Precise visualization and localization of the NVBs are often problematic during RP because of the variation in anatomical location of the cavernous nerves as well as poor exposure due to the ubiquitous presence of overlying tissues and blood during the procedure. Several studies reported that macroanatomical and electrophysiological assessments of nerve preservation showed different outcomes. The intraoperative electrophysiological assessment revealed that approximately 20% of the macroanatomical assessments were incorrect.[4] Thus, if only macroanatomical assessment was used we could not know the real impact of unilateral sural nerve interposition. With regard to the intraoperative electrophysiological test, Holzbeierlein et al claimed that responses to NVB using the CaverMap® nerve stimulator did not correlate with the precise anatomical location of the cavernous nerves as a consequence of anesthesia, medications or surgical manipulation.[13] There was a high false-negative rate reported in the CaverMap system, possibly because it measured penile tumescence. However, the most important characteristic of our system was that it measured intracavernous pressure. Kurokawa et al showed that there was no false-negative rate in this system, and that intracavernous pressure was not influenced by preoperative potency, type of anesthesia or neoadjuvant hormone therapy, suggesting high system accuracy.[6] Moreover, we revealed that nerve preservation confirmed by the system effectively predicted the recovery of potency.[14] Another important aspect of the electrophysiological assessment of NVB preservation is that the method could provide immediate feedback to surgeons during the operation. Despite attempts at direct visualization and the electrophysiological assessment of NVB, whether the preserved nerves will be functionally normal or whether vascular damage will affect continence or potency currently cannot be predicted at the time of surgery.[15] Thus, success or failure of the nerve sparing procedure is recognized 1 or 2 years after the surgery. Using the method described here a surgeon could immediately know the results of the nerve sparing procedure. This early feedback may further contribute to the improvement of the surgical outcome by the surgeon.

This prospective observational study had several limitations. This group is not a random sample and might not be representative of all men with prostate cancer who choose RP. For instance, patients electing to undergo SNG interposition may have been more motivated or surgeon technique may have changed imperceptibly with time. In addition, our study had a relatively small sample size. This poses a significant problem in the interpretation of the data because recovery of erectile function after RP depends on age as well as penile rehabilitation such that younger patients who are more motivated as seen in the nerve graft arm of this study are more likely to recover erectile function. Unfortunately our study may have insufficient power to address this issue due to the small number of patients younger than 66 years. Thus, the absence of a significant difference between the UNS+SNG group and the BNS group may be due solely to a lack of power rather than the true equivalence of these groups. We also cannot exclude RP complications such as anastomotic stricture which may impact the issue of urinary continence, although in our series the incidence was low at less than 5%. Finally, we did not distinguish those patients who use erectile aids such as phosphodiesterase type 5 inhibitors after RP. Although there is currently no consensus

regarding the implementation of penile rehabilitation programs, the initiation time, the frequency of application, the type of vasoactive agent and the dose regimen, a number of recent studies have reported on various approaches.[16,17] These factors may be a significant predictor of sexual function recovery. Despite these limitations our findings have important implications for men choosing RP for localized prostate cancer, and need to be validated in a multicenter and randomized trial.

CONCLUSIONS

These data demonstrate that in patients who underwent RP with a nerve sparing procedure, SNG interposition is associated with greater rates of postoperative sexual and urinary function. Therefore, if NVB resection is considered, SNG interposition represents an important option that may profoundly impact patient HRQOL.

Abbreviations and Acronyms

BNS	=	bilateral nerve sparing
HRQOL	=	health related quality of life
NVB	=	neurovascular bundle
PSA	=	prostate specific antigen
RP	=	radical retropubic prostatectomy
SNG	=	sural nerve graft
UCLA PCI	=	UCLA Prostate Cancer Index
UNS	=	unilateral nerve sparing

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EDITORIAL COMMENT

Enormous enthusiasm exists at present to apply cavernous nerve graft reconstruction to facilitate erectile function recovery in patients undergoing radical prostatectomy and other pelvic surgeries.[1,2] Clinical reports do indicate the feasibility of this intervention and its seemingly low morbidity. A concern is whether cavernous nerve grafting is demonstrably effective. This report does represent a fairly rigorous evaluation. The longitudinal natural history as well as the use of validated instruments are strengths. However, like other reports on this subject this study has limitations which may bias conclusions. These include lack of randomization, patient selection bias, small sample size and potential nonuniform use of erectile aids. Further study applying prospective data accrual, randomization of equally characterized, preoperatively potent patients to nerve grafting and nonnerve grafting arms of treatment, application of validated assessment tools, and sufficient clinical followup are needed for results to be conclusive.

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Sexual Function Reported by Japanese and American Men

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Purpose: We performed a cross-cultural comparison of sexual function and bother in men with localized prostate cancer in the United States and Japan.

Materials and Methods: A total of 447 Japanese and 427 American men with clinically localized prostate cancer were enrolled in separate studies of health related quality of life outcomes. Sexual function and bother were estimated before treatment with validated English and Japanese versions of the UCLA Prostate Cancer Index.

Results: Japanese men were more likely than American men to report poor sexual desire (OR 21.2, 95% CI 12.2–37.0), poor erection ability (OR 16.2, 95% CI 9.7–27.1), poor overall ability to function sexually (OR 16.7, 95% CI 9.7–28.9), poor ability to attain orgasm (OR 1.7, 95% CI 1.3–2.3), poor quality of erections (OR 2.5, 95% CI 1.9–3.5), infrequency of sexual erections (OR 2.3, 95% CI 1.7–3.1), infrequency of morning erections (OR 2.7, 95% CI 1.8–4.2) and intercourse in the previous 4 weeks (OR 2.7, 95% CI 1.9–3.8). However, Japanese men were less likely than American men to be bothered by sexual function (OR 0.36, 95% CI 0.24–0.54). A small subset of 10 Japanese-American men reported sexual function that more closely resembled their counterparts in Japan than in the United States.

Conclusions: We posit that cultural disparities in completing the quality of life surveys explain the differences in sexual activity profiles in Japanese and American men with prostate cancer.

Key Words: prostate, impotence, prostatic neoplasms, quality of life, cross-cultural comparison

Traditional definitions of the success of prostate cancer therapy have focused primarily on overall and disease-free survival, and biochemical recurrence as evidenced by PSA. Given the favorable survival outcomes associated with stage migration in recent years, patients often select primary therapy based on QOL considerations.[1] Because most men with prostate cancer are asymptomatic, it is not surprising that they are often unprepared for the diagnosis and difficult treatment decisions that they face. As treatment goals have widened to include not only survival, but also better QOL, a body of research has grown on sexual outcomes after various treatment modalities.[2,3]

Sexual function is broadly defined to include the quality and frequency of erections, strength of libido, and ability to be physically and sexually intimate, while sexual bother refers to the degree of interference or annoyance caused by any limitations in sexual function. Sexual behavior can be influenced by physiological as well as psychological or sociocultural factors.[4,5] Nonetheless, ED, which is the most common long-term side effect of prostate cancer treatment, significantly affects marital relationships.[6] Several cross-national surveys suggest that ED is more prevalent in Japanese men than in men of other countries, suggesting cultural differences in the perception and/or reporting of sexual function and associated distress.[7] However, no plausible biological explanation has been put forward.

As such, we wondered whether cross-national variations in sexual function reflect differences in the psychometric properties of the instruments rather than actual functional differences. Such a finding would have important implications for international studies that rely on instruments believed to be valid in different languages. To illuminate this issue we examined differences in self-reported sexual function and bother between American and Japanese men with newly diagnosed prostate cancer before receiving primary treatment.

MATERIALS AND METHODS

Subjects

Between 2002 and 2004, 447 Japanese men with newly diagnosed prostate cancer (cT1–T3N0M0) were enrolled in a longitudinal outcomes study. The men were treated at Tohoku University Hospital and at 2 affiliated hospitals, Kitazato University Hospital and Kurashiki Central Hospital. Between 1999 and 2003, 427 American men with localized prostate cancer (cT1–T3N0M0) who were treated with radical prostatectomy or brachytherapy at UCLA were enrolled in a separate longitudinal outcomes study. All recruitment and study protocols were approved by the institutional review boards at the respective institutions.

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Study received approval from the institutional review boards at the respective institutions.

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Outcome Measures

Sexual function and sexual bother (distress from sexual dysfunction) were measured separately with the UCLA PCI.[8] Each UCLA PCI domain is scored from 0 to 100 points with higher scores representing better outcomes. Hence, a higher sexual function score indicates better function and a higher sexual bother score indicates less bother. On the UCLA PCI sexual function is measured by 8 items and sexual bother is measured by a single item. UCLA PCI performs well not only in men with prostate cancer, but also in healthy older man without prostate cancer[9] and in men presenting for evaluation for ED.[10] A validated Japanese version is available.[11]

Questionnaires were completed at home within the month before surgery and returned in postage prepaid envelopes. All men were aware of the cancer diagnosis before completing the questionnaire. Comorbidities were documented using a patient reported checklist at baseline, while clinical variables were abstracted from medical records.

Statistical Analysis

Descriptive statistics are presented for demographic and clinical characteristics. We dichotomized the response to each item, grouping the best and worst outcomes (see Appendix). We used the chi-square test to compare the 9 items between Japanese and American men. We also performed logistic regression for each item with country as our main predictor variable, adjusting for each subject age with $p < 0.05$ considered statistically significant. All statistical analyses were performed with SAS® 9.1.

TABLE 1. *Sample characteristics*

	Japanese		American		p Value
No. pts	412		427		
Age at survey:					<0.001
Mean ± SD	67.2 ± 5.5		62.1 ± 7.9		
Median (range)	67	(47–81)	62	(40–83)	
PSA at diagnosis (ng/ml):					
Mean ± SD	8.7 ± 5.9		8.0 ± 9.0		0.194
Median (range)	7.1 (1.3–54)		6 (0.02–120)		
No. clinical stage (%):					<0.001
T1	239	(58)	305	(71)	
T2	148	(36)	117	(28)	
T3	25	(6)	5	(1)	
No. initial treatment (%):					0.001
Radical prostatectomy	350	(85)	325	(76)	
Brachytherapy	62	(15)	102	(24)	
No. biopsy Gleason sum (%):					<0.001
6 or Less	171	(42)	303	(71)	
7 or Greater	241	(58)	121	(28)	
Unknown	—		3	(1)	
No. comorbidity count (%):*					0.002
0	152	(37)	157	(37)	
1	113	(27)	156	(37)	
2	77	(19)	70	(16)	
3 or Greater	70	(17)	42	(10)	
Unknown	—		2	(0)	
No. ethnicity (%):					—
White	—		352	(82)	
Black	—		14	(3)	
Hispanic	—		13	(3)	
Asian	412	(100)	18	(4)	
Multiracial	—		5	(1)	
Other	—		24	(6)	

* Including hypertension, stomach, intestinal and gastrointestinal diseases, heart disease, cancer other than prostate cancer, lung disease, diabetes, stroke and blood disease.

RESULTS

Surveys were received from 412 Japanese and 427 American men. None of the men received androgen ablation before completing the questionnaire. The American men were younger and had earlier stage and lower grade tumors ($p < 0.001$, table 1). The groups did not differ in mean prebiopsy PSA.

Even after controlling for age the Japanese men were much more likely than American men to report low sexual desire (OR 21.2, 95% CI 12.2–37.0), poor erections (OR 16.2, 95% CI 9.7–27.1) and poor overall sexual function (OR 16.7, 95% CI 9.7–28.9). The Japanese men were more likely to report difficulty with orgasms (OR 1.7, 95% CI 1.3–2.3), and low quality (OR 2.5, 95% CI 1.9–3.5) and frequency (OR 2.3, 95% CI 1.7–3.1) of erections. The Japanese men were more likely to report not having morning erections (OR 2.7, 95% CI 1.8–4.2) and report having achieved no intercourse in the previous 4 weeks (OR 2.7, 95% CI 1.9–3.8). However, despite reporting worse sexual function for all 8 items the Japanese men were significantly less likely to report sexual bother (OR 0.36, 95% CI 0.24–0.54, table 2). When analysis was limited to men 60 years or younger in the 2 ethnic groups, the differences persisted for sexual function (items 1 to 8) but not for sexual bother (item 9).

Of the American cohort 18 men were Asian, including 10 who were identified by their surnames as Japanese-Americans. We performed an exploratory ad hoc analysis in these 10 Japanese-American men to identify potential acculturation effects. Nine of the 10 Japanese-American men reported that sexual desire, erection ability, orgasm and awakening with an erection were fair, poor or very poor. Furthermore, all 10 men reported that overall sexual function ability was poor or very poor. Six of the 10 men considered that the quality and frequency of erections were poor or very poor. Only 2 of the 10 men reported having achieved intercourse more than once during the previous 4 weeks. The Japanese-American men reported dysfunction at rates closely approximating those of the native Japanese men but these 10 men reported almost twice as much distress from sexual function as the Japanese men (60% vs 36%).

DISCUSSION

Our study has several important findings. 1) We found different cultural profiles of sexual function in Japanese and American men with localized prostate cancer. Using a self-reported questionnaire Japanese men reported less sexual activity than American men even after adjusting for age. This finding is consistent with other reports, in which ED and decreased libido were noted in a greater proportion of Japanese than American men.[12] Population based data from Japan indicate that the proportion of ED is 20%, 42% and 64% for ages 50 to 59, 60 to 69 and 70 to 79 years, respectively, which are higher than in other countries.[4,13] The relationship between sexual function and serum testosterone is controversial. However, in the Japanese male population no continuous decrease in testosterone has been noted after age 40 years, as it has in other countries.[14]

In Japan physician consultation time at outpatient clinics is much shorter than in the United States. Moreover, the condition of the clinical setting at Japanese hospitals may decrease patient motivation. Some patients are asked to

TABLE 2. *Men reporting poor sexual function by ethnicity*

Question No.	Question	% Japanese (No.)	% American (No.)*	OR (95% CI)
		412	427	
1	Sexual desire level	96 (394)	51 (214)	21.2 (12.2–37.0)
2	Ability to achieve erection	95 (386)	49 (204)	16.2 (9.7–27.1)
3	Ability to achieve orgasm	62 (246)	40 (167)	1.7 (1.3–2.3)
4	Erection quality	64 (258)	33 (137)	2.5 (1.9–3.5)
5	Erection frequency	61 (246)	32 (135)	2.3 (1.7–3.1)
6	Awakened with erection	91 (367)	72 (303)	2.7 (1.8–4.2)
7	Intercourse	83 (335)	56 (238)	2.7 (1.9–3.8)
8	Overall sexual function ability	96 (387)	52 (216)	16.7 (9.7–28.9)
9	Sexual function problem	11 (47)	25 (97)	0.36 (0.24–0.54)

All models were controlled for patient age.
* Referent.

wait outside the consultation room but they can overhear conversations between the doctor and other patients, which may in turn cause them to be self-conscious about discussing sensitive issues with the physician during their own consultations. These factors in the clinical setting discourage patients from raising complicated psychological issues, such as sensitive sexual aspects of treatment, and force them to focus on the general physical aspects. A safer and more private atmosphere might encourage more open dialogue about sexual function. Nonetheless, it would be valuable to distinguish between patients who do not put a high priority on sexual function and those who are hesitant to raise the topic with their medical providers. Conversely while male erectile rigidity contributes to the frequency of sexual intercourse, it is not necessarily associated with a satisfactory sexual life in the partners of Japanese men.[15] The discrepancy between the responses of Japanese males and their partners might be explained by discordant views of what represents a satisfactory sexual life, eg noncoital intimate activities. That the lack of privacy during consultations inhibits frank personal discussions may also limit candor in written self-reported sexual function.

2) Although Japanese men reported less sexual activity than American men, Japanese men were less likely to be bothered. Other studies show that, unlike their American counterparts, older Japanese men do not report dissatisfaction with sexual life.[11] The pattern of help seeking behaviors differs substantially between Japan and the United States. In Japan most men take no action, while in America men may seek help from the partner, family members or other sources of social support. Of those who do not seek treatment younger men seem to believe that ED would resolve spontaneously, while older men resist seeking treatment because they believe that ED is a natural part of aging.[14]

Although Japanese beliefs regarding sexual dysfunction have changed considerably in recent years, discussion of sexually related topics continues to be repressed in Japanese patient-physician encounters. In fact, the most commonly cited reason for not self-referring to a doctor is that sexual problems are not medical problems.[16] In Japan it may be difficult to find medical professionals who consider it their role to deal with the sexual issues of a patient. Hence, in addition to improving privacy during consultations, it is also important that providers be trained more comprehensively in male health. This added emphasis would focus greater attention on the sexual concerns of cancer survivors, especially given the recent pharmacological advances in this discipline.

Conversely American men tend to perceive sexual difficulties as a serious medical issue that requires intervention. This is consistent with other reports documenting the great weight that sexual dysfunction carries with many American men.[2,4] Being married or living with a partner might motivate American men to seek treatment for ED, which caused more distress due to decreased function.

Although our Japanese-American sample was too small for statistical analyses, several observations in this group are noteworthy and hypothesis generating. In particular the patterns of sexual function reported by Japanese-American men were remarkably similar to those reported by Japanese men. This is consistent with the relatively low reported rates of sexual assertiveness in Asian countries.[17] Contributing to this lower reported sexuality are Asian cultural restraints on sexual behavior. This suggests that cultural factors and deeply embedded health beliefs may have a decisive role in defining health seeking behaviors for sexual problems. However, to our knowledge there have been no empirical investigations of the specific role of culture in Japanese-American men. However, despite similar sexual function Japanese-American men in our sample showed a trend toward more sexual bother than their Japanese counterparts. The reason for this discrepancy is unclear but it may be related to the natural evolution of cultural norms in immigrant communities. The Japanese-American men may have perceived sexual dysfunction more as a medical issue than did their peers in Japan.[17] Future research is needed to investigate the cultural and structural causes of this variation.

There are several important limitations to this study. 1) This group is not a random sample and it might not be representative of all men with newly diagnosed early prostate cancer. Selection bias may have been introduced by including individuals who were more interested in the topic or had more time to answer the questions. Moreover, the groups were not well balanced in several domains, such as age, treatment type and comorbidity. 2) We did not distinguish men who may have used erectile aids, such as type 5-phosphodiesterase inhibitors or vacuum devices. These factors may limit the generalizability of our findings. 3) Health related QOL and patient satisfaction may depend in part on factors such as the content of counseling, which are more difficult to measure. 4) Because we assessed outcomes after the cancer diagnosis, sexual function and bother outcomes may have been affected even before treatment. However, other investigators using the UCLA PCI noted no differences in sexual domain scores before

and after diagnosis.[18] 5) The lack of objective data, such as serum testosterone and audiovisual sexual stimulation tests, was inherent in our study design.

Despite these limitations to our knowledge this cross-national survey is the first to document such differences in a prostate cancer population. It may increase physician awareness and understanding of sexual health issues, and help them encourage patients to identify and overcome potential barriers to discussing and seeking help for sexual dysfunction. Different cultures have different concepts of health, including sexuality, well-being, illness and disease. Even using validated survey instruments we must be aware that multicultural issues may result in significant bias in data collection. Further research is required to understand fully which factors are most important in the individual ethnicities.

CONCLUSIONS

In patient reported questionnaires Japanese men with localized prostate cancer report worse sexual function and less bother from the decreased function than American men. Cultural differences appear to have an important role in the reporting of sexual activity and the perception of related distress. In the absence of an underlying biological explanation for cross-national differences in sexual function we suspect that cultural differences in how the QOL surveys were interpreted may explain the differences in sexual activity profiles in Japanese and American men with prostate cancer.

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APPENDIX

Definitions of Poor Sexual Function

1. Your level of sexual desire?
   Very poor/poor/fair vs good/very good
2. Your ability to have an erection?
   Very poor/poor/fair vs good/very good
3. Your ability to reach orgasm (climax)?
   Very poor/poor/fair vs good/very good
4. How would you describe the usual QUALITY of your erection?
   None at all/not firm enough for any sexual activity/firm enough for masturbation or foreplay only vs firm enough for intercourse
5. How would you describe the FREQUENCY of your erections?
   Never/less than half/about half the time I wanted vs more than half the time I wanted/whenever I wanted
6. How often have you awakened in the morning or night with an erection?
   Never/seldom (less than 25% of the time)/not often (less than half the time) vs often (more than half the time)/very often (more than 75% of the time)
7. During the last 4 weeks did you have vaginal or anal intercourse?
   No/once vs more than once
8. Overall, how would you rate your ability to function sexually during the last 4 weeks?
   Very poor/poor/fair vs good/very good
9. Overall, how big a problem has getting and maintaining an erection been for you during the last 4 weeks?
   Big problem/moderate problem vs small problem/very small problem/no problem

Abbreviations and Acronyms

ED = erectile dysfunction
PSA = prostate specific antigen
QOL = quality of life

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Glycosylation status of haptoglobin in sera of patients with prostate cancer *vs.* benign prostate disease or normal subjects

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We studied chemical level and glycosylation status of haptoglobin in sera of patients with prostate cancer, as compared to benign prostate disease and normal subjects, with the following results. (*i*) Haptoglobin level was enhanced significantly in sera of prostate cancer. (*ii*) Sialylated bi-antennary glycans were the dominant structures in haptoglobins from all 3 sources, regardless of different site of N-linked glycan. The N-linked glycans at N184 were exclusively bi-antennary, and showed no difference between prostate cancer *vs.* benign prostate disease. (*iii*) Tri-antennary, N-linked, fucosylated glycans, carrying at least 1 sialyl-Lewis$^{x/a}$ antenna, were predominantly located on N207 or N211 within the amino acid 203-215 sequence of the β-chain of prostate cancer, and were minimal in benign prostate disease. Fucosylated glycans were not observed in normal subjects. A minor tri-antennary N-linked glycan was observed at N241 of the β-chain in prostate cancer, which was absent in benign prostate disease. (*iv*) None of these N-linked structures showed the expected presence of disialylated antennae with GalNAcβ4(NeuAcα3)Galβ3(NeuAcα6)GlcNAcβGal, or its analogue, despite cross-reactivity of prostate cancer haptoglobin with monoclonal antibody RM2. (*v*) Minor levels of *O*-glycosylation were identified in prostate cancer haptoglobin for the first time. Mono- and disialyl core Type 1 O-linked structures were identified after reductive β-elimination followed by methylation and mass spectrometric analysis. No evidence was found for the presence of specific RM2 or other tumor-associated glycosyl epitopes linked to this *O*-glycan core. In summary, levels of haptoglobin are enhanced in sera of prostate cancer patients, and the *N*-glycans attached to a defined peptide region of its β-chain are characterized by enhanced branching as well as antenna fucosylation.

Key words: *N*-glycosylation; tri-antennary; haptoglobin; beta-chain; alpha2-chain; glycopeptides; antenna fucosylation

Aberrant glycosyl epitopes, defined by specific monoclonal antibodies (mAbs), in glycolipids and glycoproteins have been regarded as useful biomarkers for cancer diagnosis,[1] and in some cases for cancer therapy[2–4]; for review see Ref. 5. Out of many glycosyl epitopes, lacto-series Type 1 chain structures Le^c,[6,7] sialyl-Le^a,[8] disialyl-Lc4,[9] and dimeric-Le^a,[10,11] and O-linked Tn[12,13] and sialyl-Tn,[14,15] have often been regarded as useful biomarkers. The antigen GalNAcβ4-disialyl-Lc4,[16] defined by mAb RM2, was found to react with prostate cancer tissue, but not with benign prostate disease or normal prostate gland.[17]

Haptoglobin, a hemoglobin-binding glycoprotein, is a major serum component, consisting of β-, α1- and α2-chains, which are disulfide-linked together in various combinations. Differences in subunit combination pattern give rise to a large variety of haptoglobin polymorphism patterns in serum. In addition, the haptoglobin chemical level in serum increases significantly in association with inflammation, cancer development, and some physiological processes (for review see Refs. 18 and 19).

While there are 4 consensus sequences for *N*-glycosylation sites in the β-chain of haptoglobin, none is located in the α-chain. During the past decade, the *N*-glycosylation status of haptoglobin, with particular focus on changes of fucosylation and sialylation associated with many diseases has been extensively studied, since haptoglobin is regarded as an "acute-phase protein" and is elevated in many diseases (see Discussion).

In view of recent progress, particularly with glycomics approach by mass spectrometry, regarding glycosylation status of haptoglobin associated with various types of cancer (see Discussion), and the lack of such studies on prostate cancer haptoglobin, we performed the following studies: (*i*) Comparison of haptoglobin levels in sera of prostate cancer patients with levels in benign prostate disease and normal subjects. (*ii*) Mass spectrometric analysis of tryptic glycopeptides obtained from haptoglobin which was affinity-purified through a hemoglobin-Sepharose column. (*iii*) Mass spectrometric analysis of N-linked glycans released by PNGase-F from haptoglobin β-chain. (*iv*) Mass spectrometric analysis of O-linked glycans released by reductive β-elimination of affinity-purified haptoglobin.

Our results indicate that N-linked, tri-antennary glycans carrying 1 or more SLe$^{x/a}$ antennae in 1 *N*-glycosylation domain of the haptoglobin β-chain are highly expressed in prostate cancer patients, but minimal in benign prostate disease or normal subjects. Additionally, we show that prostate cancer haptoglobin carries a low level of *O*-glycosylation, but that no specific glycosyl epitope such as RM2 or its analogue is detectable by the mass spectrometric methods employed, in either N-linked or O-linked glycans in the β- or α-chain of haptoglobin.

Abbreviations: aoWR, N^{α}-((aminooxy)acetyl)tryptophanylarginine methyl ester; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; PBS (-), phosphate-buffered saline (-), *i.e.*, 0.15 M NaCl, 20 mM of a mixture of Na_2HPO_4/NaH_2PO_4, pH adjusted to 7.2, without addition of Ca^{2+} or Mg^{2+}); PNGase-F, peptide-*N*4-(acetyl-β-glucosaminyl)-asparagine amidase; RM2 antigen, GalNAcβ4-disialyl-Lc4; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SLe^a, sialyl-Lewis^a; SLe^x, sialyl-Lewis^x.

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Material and methods

Materials

Anti-human haptoglobin rabbit antibodies were from Dako (Carpinteria, CA). Peroxidase-coupled goat anti-rabbit IgG, used as secondary antibody was from Santa Cruz Biotech (Santa Cruz, CA). Chemiluminescent detection system (Super Signal West Pico) was from Pierce Biotechnology (Rockford, IL).

General procedure: Steps for serum collection and haptoglobin analysis

Step 1. Sera from patients with prostate cancer, or benign prostate disease, were collected based on diagnosis of patients by biopsy with histological examination by expert clinical pathologist, at the Dept. of Urology, Tohoku University School of Medicine, and sent in frozen state to Pacific Northwest Research Institute (PNRI), Seattle, WA, USA. The Ethics Committees of both Tohoku University and PNRI approved the study. PSA values of sera were also recorded (see below). PSA value (ng/ml) is given in parenthesis after case number.

a. Sera from prostate cancer. Case 5 (48.7); Case 21 (7.2); Case 23 (12.8); Case 32 (3.9); Case 40 (5.8); Case 71 (5.6); Case 72 (5.2); Case 76 (2.0).
b. Sera from benign prostate disease: Case 6 (7.3); Case 33 (3.0); Case 43 (9.2); Case 77 (13.2); Case 92 (3.9); Case 105 (13.6); Case 137 (4.1); Case 150 (6.6).

Serum volume sent from Tohoku University was 0.5–1.0 ml for each case. Sera from 8 cases each of prostate cancer and benign prostate disease were respectively combined and used for preparation of haptoglobin β-chain and α2-chain followed by examination of *N*- and *O*-glycosylation status. Sera from 8 normal subjects (volunteer workers) were prepared at PNRI.

Step 2. Combined sera as above containing equal protein quantity, or sera from normal subjects, were used for (*i*) determination of level of haptoglobin β-chain and α2-chain by Western blot analysis followed by densitometry of each band; (*ii*) affinity separation of haptoglobin by hemoglobin conjugated to Sepharose column; (*iii*) separation and purification of β-chain and α-chain, after cleavage of disulfide bonds by reductive alkylation. Procedures (*i*), (*ii*) and (*iii*) were performed at PNRI, Seattle, WA. Purified haptoglobin β-chain was sent to both Laboratory of Advanced Chemical Biology, Hokkaido University, Japan, and to Division of Molecular Biosciences, Imperial College London, UK.

Step 3. (*i*) Separation of tryptic glycopeptide-1, -2 and -3, covering each *N*-glycosylation site of haptoglobin β-chain, and determination of their structures, and (*ii*) determination of N-linked glycan structure released from each tryptic glycopeptide by PNGase-F (Roche Applied Science), were performed by the Hokkaido University group. (*iii*) In addition, total N-linked glycan of purified haptoglobin β-chain released by PNGase-F directly applied on haptoglobin β-chain, with particular focus on structure of fucosylation at antenna, was performed by the Imperial College London group.

Step 4. Structures of O-linked glycans from purified haptoglobin released by reductive β-elimination, and presence or absence of any known glycosyl epitope such as RM2 epitope, were rigorously studied by the Imperial College London group.

Serum haptoglobin levels

Step 2-(i). Individual sera (12 from prostate cancer cases, 9 each from benign prostate disease and from normal subjects) were used for determination of haptoglobin levels. An aliquot of serum containing equal 0.25-μg protein from each case was subjected to Western blot with anti-human haptoglobin rabbit antibodies, followed by secondary goat anti-rabbit IgG, and finally chemiluminescent detection system, as described previously.[20] The protein level of each serum sample was determined using a Micro-BCA protein assay reagent kit (Pierce).

Intensities of bands for β-chain and α2-chain from normal subjects, benign prostate disease, and prostate cancer were determined by densitometry with Scion Image program, as described in the Figure 1*a* legend.

Purification of haptoglobin and separation of β-, α2- and α1-chains

Step 2-(ii). Preparation of human serum haptoglobin by hemoglobin-affinity column. Human serum haptoglobin was prepared by affinity chromatography using freshly prepared human hemoglobin conjugated to Sepharose 4B, by the cyanogen bromide activation method as described previously.[21] Combined sera (~2–3 ml) were subjected to hemoglobin-Sepharose 4B column (1.0 × 18 cm^2, volume 14 ml) prepared in PBS (-) medium, pH 7.2 (see Abbreviations). An LKB peristaltic pump was used, and the flow rate was 0.5 ml/min. The eluent was monitored at 280 nm using a Waters 484 Tunable absorbance detector. A Gilson fraction collector model 203 was used, and 5-ml fractions were collected.

Applying human serum on the hemoglobin affinity column, most of the serum proteins passed through with PBS (-) wash, which was followed by washing with 0.15 M NaCl, pH 11 to elute apo AI. Finally, haptoglobin was eluted with 5 M urea in 0.15 M NaCl pH 11 solution. The eluate for haptoglobin was desalted and concentrated with an Amicon Centricon Plus-70 (Millipore) using Beckman centrifuge model J-6M at 3,000*g* for 10 min. The concentrated solution was diluted several times with 30-ml pure water, reconcentrated to remove urea, and finally lyophilized. Total haptoglobin fraction from ~2–3 ml serum, adsorbed on and eluted from hemoglobin column as above, was ~200–250 μg.

Step 2-(iii). Separation of haptoglobin β-, α2- and α1-chains by reductive alkylation followed by gel filtration. Haptoglobin purified as above is a hetero-tetramer cross-linked with inter and intra S–S bridges $(\alpha\beta)_2$. The subunits were separated by reductive alkylation with acrylamide as described previously.[20,22] Briefly, crude haptoglobin (~200 μg) was dissolved in 100 mM NH_4HCO_3, pH 8.5, and mixed with a vortex. Dithioerythritol was added to a final concentration of 150 mM and the pH was adjusted to 8.3 using 1 N NaOH before incubation at 37°C for 1 hr under nitrogen to cleave the S–S cross-links. Alkylation of the thiol groups was performed with 300 mM acrylamide to form propionamide cysteine (PAM) derivatives. The alkylation reaction was stopped on ice and the mixture was filtered with Millipore Ultrafree-MC (0.45 μm).

Separation of alkylated haptoglobin β-, α2- and α1-chains was performed by gel filtration through Superdex200™ 10-300GL (1.0 × 30 cm^2, bed volume 24 ml), in 50 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS (-)). The HPLC (Waters 490) column was attached to a Waters UV detector and a Gilson fraction collector (model 203) was used. The flow rate was 0.4 ml/min. Each subunit was collected as 1 fraction and dialyzed using Spectra/Por molecular porous membrane tubing (MWCO 3.500, diameter 34 mm) (Spectrum Laboratories, Rancho Dominguez, CA) against 0.1% acetic acid for 40–50 hr. The solution was then transferred to an Eppendorf tube (1.5 ml) and lyophilized.

N-linked and O-linked carbohydrate analysis by mass spectrometry

Step 3-(i). Separation of tryptic glycopeptide-1, -2 and -3, covering each N-glycosylation site of haptoglobin β-chain, and determination of their structures by MALDI-TOF MS. Purified haptoglobin β-chain (~2 μg) from prostate cancer, benign prostate disease and normal subjects, respectively, was subjected to quantitative *N*-glycan profiling analysis, at the Laboratory of Advanced Chemical Biology, Hokkaido University. Each haptoglobin β-chain was reductively alkylated and digested with

trypsin, and *N*-glycans were enzymatically liberated from each haptoglobin β-chain using PNGase-F, and purified by a combination of methods previously described, with some modifications.[23–25] The sialic acid residue(s) of the *N*-glycans were methylesterified,[26] and labeled with aoWR.[27] For analyses of glycopeptides having N-linked glycan, purified haptoglobin β-chain (4–8 µg) was dried by centrifuge concentrator (Speed-Vac, Savant), and solubilized with 50 mM ammonium bicarbonate (pH 7.8) at a concentration of 0.2–0.4 mg/ml. Samples were digested with 0.5 µg modified trypsin at 37°C for 2 hr. The reaction was terminated by heating at 100°C for 3 min. The tryptic glycopeptides (glycopeptide-1, -2 and -3; see Results) derived from haptoglobin β-chain were purified with hydrophilic affinity isolation and reverse phase HPLC technique as described previously.[28] Briefly, the tryptic digest was mixed with a 15-µl packed volume of Sepharose 4B in 1 ml of an organic solvent of 1-butanol/ethanol/H_2O (4:1:1, v/v). After gentle shaking for 45 min, the gel was washed twice with the organic solvent, and then incubated with an aqueous solvent of ethanol/H_2O (1:1, v/v) for 30 min. The solution phase was recovered, dried and further fractionated by HPLC on a C18 column with a linear grandient elution of acetonitrile (5–50%, v/v) in 0.1% (v/v) trifluoroacetic acid. The glycopeptide fractions were mixed with 2,5-dihydroxybenzoic acid (10 mg/ml in water) and subjected to linear TOF measurements using an Ultraflex TOF mass spectrometer equipped with pulsed ion extraction system (Bruker Daltonik GmbH, Bremen, Germany). The ions generated by a pulsed UV laser beam (nitrogen laser, 337 nm, 10 Hz) were accelerated to a kinetic energy of 25.0 kV.

Step 3-(ii). MS and MS/MS analysis of permethylated N- and O-glycans from prostate cancer haptoglobin. A sample of prostate cancer haptoglobin (20 µg) was reduced for 1 hr at 37°C in 50 mM Tris-HCl buffer (pH 8.5) containing a 4-fold excess of dithiothreitol and carboxymethylated with a 2-fold molar excess of iodoacetic acid for 1 hr at room temperature in the dark. Following dialysis at 4°C for 72 hr against 4 × 4.5 l of cold 50 mM ammonium bicarbonate, pH 7.5, and lyophilization, the sample was digested with sequencing-grade trypsin (Promega) (1 µg in 50 mM ammonium bicarbonate, pH 8.5, for 18 hr at 37°C). The reaction was stopped by adding a few drops of acetic acid to the solution. The sample was lyophilized, dissolved in 150 µl (5% (v/v)) acetic acid, and purified using a SepPak cartridge C_{18} (Waters), as described.[29] The purified glycopeptides were digested with PNGase-F in 50 mM ammonium bicarbonate (pH 8.5) containing 10 U of enzyme at 37°C over 18 hr. The sample was lyophilized, and the released *N*-glycans were purified using a SepPak cartridge C_{18} (Waters Corp.). Permethylation and sample clean-up were performed using the sodium hydroxide protocol, as described previously.[29]

Steps 3-(iii) and -(iv). For *O*-glycan analysis, fractions from the gel filtration column corresponding to the α2- and β-chains (see above and Fig. 1*c*) were subjected to reductive elimination by adding 400 ml of 1 M potassium borohydride (dissolved in 0.1 M potassium hydroxide) for 24-hr incubation at 45°C. The reaction was stopped by adding a few drops of acetic acid. Further cleaning of the removed glycans was achieved by Dowex beads minicolumn purification followed by borate removal using 10% of methanolic acetic acid.[29] Permethylation and sample clean-up were performed using the sodium hydroxide protocol, as described previously.[29]

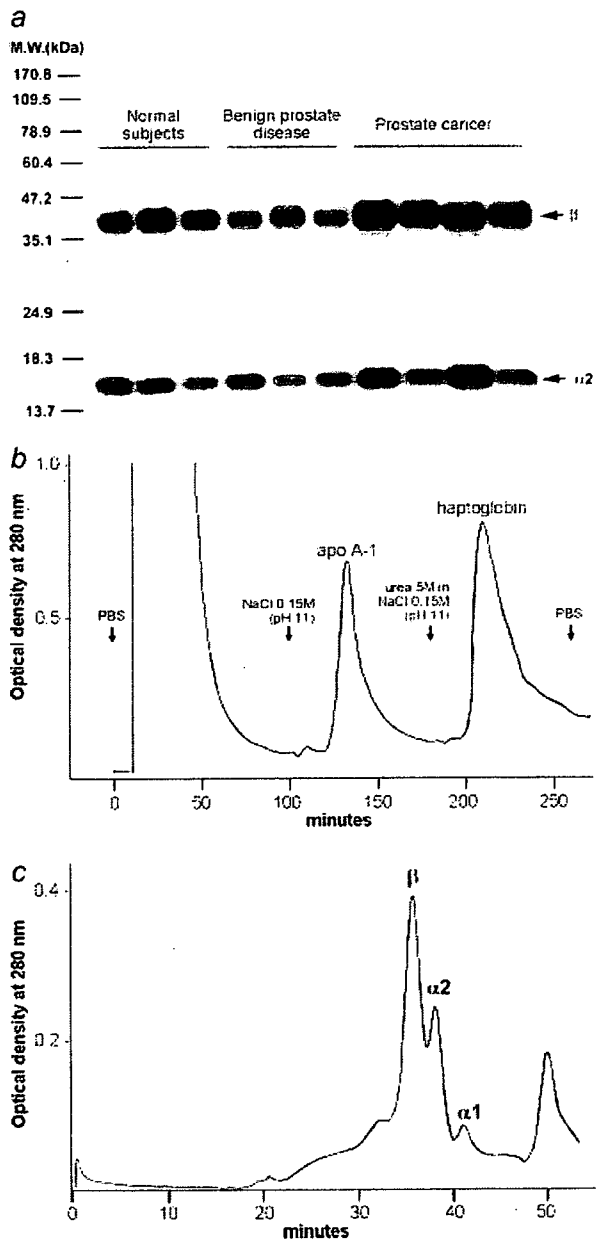

FIGURE 1 – Pattern of serum haptoglobin, affinity purification, and separation of β-, α1- and α2-chains. (*a*) Pattern of haptoglobin β-chain and α2-chain separated by 1-D SDS-PAGE, with Western blot with anti-haptoglobin antibodies. In total, 9 cases of normal subjects, 9 cases of benign prostate disease, and 12 cases of prostate cancer were analyzed. The figure shows 3 cases of normal subjects, 3 cases of benign prostate disease, and 4 cases of prostate cancer. For summary of analysis, see Table I. 0.25-µg protein from each serum was separated on 1-D SDS-PAGE, as described previously.[20] Proteins on a gel were blotted onto Immobilon P using a tank type apparatus from Nihon Eido (Tokyo, Japan) at 45 V for 50 min, in trans-blot buffer containing 10 mM CAPS and 10% methanol at pH 11. The membranes were blocked with 5% skim milk for 2 hr and incubated overnight with primary anti-human haptoglobin rabbit antibody (Dako). Peroxidase-coupled goat anti-rabbit IgG was used as secondary antibody. Finally, chemiluminescent detection system (Super Signal West Pico, Pierce Biotechnology) was used to enhance the bands or spots stained. (*b*) Affinity chromatography of haptoglobin from sera of prostate cancer patients. About 5 ml of serum from prostate cancer patients was loaded on hemoglobin-Sepharose 4B affinity column. (*c*) Separation of haptoglobin β-, α1- and α2-chains by gel filtration through Superdex200™ 10-300GL column. Crude haptoglobin (~200 µg) was reduced with dithioerythritol and alkylated with acrylamide. Experimental conditions for *b* and *c*: see Material and methods.

TABLE I – DENSITOMETRIC DETERMINATION OF WESTERN BLOTTED BANDS OF HAPTOGLOBIN β-AND α2-CHAINS BY ANTI-HAPTOGLOBIN ANTIBODIES IN 9 CASES EACH OF NORMAL SUBJECTS AND BENIGN PROSTATE DISEASE, AND 12 CASES OF PROSTATE CANCER

	n	mean ± SD	Significance of difference
β-chain			
Normal	9	8049 ± 2063	$p < 0.01$
Benign prostate	9	6866 ± 1647	
Prostate cancer	12	13462 ± 1487	
α2-chain			
Normal	9	8108 ± 2767	$p < 0.01$
Benign prostate	9	7673 ±1825	
Prostate cancer	12	13084 ± 3064	

MALDI-TOF MS data on permethylated samples were acquired at Imperial College London using a Perseptive Biosystems Voyager DE-STR™ mass spectrometer in the reflector mode with delayed extraction. MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV, and argon was used as collision gas. Samples were dissolved in 10 µl methanol and mixed at a 1:1 ratio (v/v) with 2,5-dihydrobenzoic acid as matrix.

Results

Haptoglobin levels in sera of prostate cancer patients, benign prostate disease and normal subjects

Haptoglobin levels in sera of prostate cancer patients were significantly higher than those from benign prostate disease or normal subjects, as determined by Western blot analysis of both β- and α2-chain with anti-haptoglobin antibodies (Fig. 1*a*). Mean densitometric values for 12 cases of prostate cancer, compared to 9 cases of benign prostate disease, and 9 normal subjects, for β-chain, were 1.9 and 1.7 times higher, respectively. Values for the α2-chain were 1.7 and 1.6 times higher, respectively. Each of these differences was statistically significant ($p < 0.01$), as indicated (Table I).

Separation of haptoglobin into its β-, α2- and α1-chains

To search for cancer-associated glycosyl epitopes in haptoglobin we chose to isolate its subunits and search for glycopeptides after tryptic digestion.

Thus haptoglobin from sera of prostate cancer patients, benign prostate disease, or normal subjects was affinity purified on a hemoglobin-Sepharose 4B column and the β-, α2- and α1-chains were separated after reductive alkylation, as detailed in Material and methods. A typical example of the affinity chromatography pattern is shown in Figure 1*b*, and a typical example of the gel filtration pattern of the β-, α2- and α1-chains is shown in Figure 1*c*. Once again, we could observe that the quantities of the β- and α2-chain in prostate cancer patients were significantly higher than those in benign prostate disease or normal subjects (Table I).

Glycomics profiling of the β-chain reveals enhanced fucosylation in prostate cancer

A similar quantity of purified haptoglobin β-chain (*e.g.*, ~2 µg per analysis) from prostate cancer, benign prostate disease and normal subjects was subjected to PNGase-F digestion. The released N-linked glycans were purified from a crude digestion mixture by glycoblotting technique through reaction of the reducing end of the carbohydrate with an amino-oxime group or hydrazide group affixed on solid phase.[23] To perform quantitative MALDI-TOF analysis with high sensitivity, the captured glycans were further methylesterified at the carboxyl group of sialic acid residue(s),[26] and were derivatized with aoWR.[27]

Spectra of the N-linked glycans obtained from the β-chain of prostate cancer, benign prostate disease and normal subjects are shown in Figure 2. As shown in the annotations, the major glycans shared by all 3 samples are mono- and disialylated bi-antennary

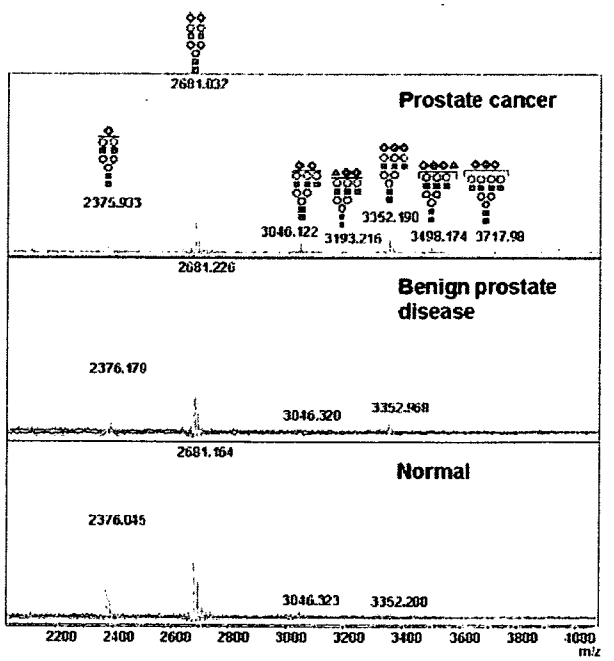

FIGURE 2 – MALDI-TOF spectra showing differences in N-linked glycans of haptoglobin β-chain from sera of prostate cancer, benign prostate disease, and normal subjects. Glycans were released by PNGase-F, methylesterified at the carboxyl group of sialic acid(s), and derivatized with aoWR. Keys are given in the box and correspond to Consortium for Functional Glycomics symbols (http://www.functionalglycomics.org/fg/). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

structures (theoretical *m/z* 2,375.9 and 2,681.0). All 3 samples also contain a minor trisialylated tri-antennary glycan, the molecular ion at *m/z* 3,352.2 and 3,352.9, which is much more abundant in prostate cancer than in benign prostate disease or normal subjects. A tiny amount of disialylated tri-antennary glycan, the molecular ion at *m/z* 3,046.2, is observed in the prostate cancer and benign prostate disease samples. The most striking difference between the samples is the presence of *m/z* 3,498.2 in the prostate cancer spectrum (Fig. 2, upper panel). This signal, which is absent in the spectra from benign prostate disease and normal subjects (Fig. 2, middle and bottom panels), corresponds to a mono-fucosylated tri-antennary glycan, the molecular ion at *m/z* 3,498.2.

Fucosylated tri-antennary N-glycans are specific to one of the glycosylation domains of the β-chain

The consensus sites for *N*-glycosylation are Asn-184 (N184), N207, N211 and N241. To investigate the location of the fucosylated *N*-glycans observed in the glycomic profiling, the β-chain samples from prostate cancer, benign prostate disease, and normal subjects were subjected to tryptic digestion and MALDI analysis. Tryptic digestion was predicted to yield 3 glycopeptides: (*i*) 203-215 carrying 2 glycosylation sites, N207 and N211, (*ii*) 236-251 carrying 1 glycosylation site at position N241 and (*iii*) 179-202 carrying 1 glycosylation site at position N184. The resulting glycopeptides were fractionated by successive hydrophilic affinity isolation and reversed-phase HPLC, and were analyzed by MALDI-TOF mass spectrometry. Each glycopeptide yielded a cluster of molecular ions because of glycan heterogeneity. Under the chromatographic conditions employed, we observed that glycopeptides of different glycoforms on the same peptide tended to elute in close proximity on reversed-phase chromatography analyses. The relative microheterogeneity of different glycoforms present at a particular *N*-glycosylation site(s) was determined by com-