(bcl2 first forward), TTCCAGATCGATTCCCAGAC (bcl2 first reverse), CGTCCTGCCTTCATTTATCC (bcl2 second forward) and TTCGCAGAAGTCCTGTGATG (bcl2 second reverse). PCR reactions were done in a total volume of 20 μl consisting of 0.3 μl 10 μmol/l solution of each primer, 1.5 mmol/l $MgCl_2$, 0.8 mmol/l deoxynucleotide triphosphate, 0.5 U REDTaq® DNA polymerase, 1 μl (80 ng/μl) genomic DNA and 15.6 μl H_2O using a PTC 200 Thermal Cycler (MJ Research, Waltham, Massachusetts). All reactions were subjected to 2 amplification rounds using a nested primer approach. The first and second PCR annealing temperatures and PCR cycles were 52C and 48 cycles, and 58C and 45 cycles, respectively. The second PCR products (195 bp), including the polymorphic site, were digested with the restriction enzyme *BccI* (New England BioLabs®) at 37C for 3 hours, separated on 2.0% agarose gel and stained with ethidium bromide. Unrestricted products (195 bp) represented the C/C genotype and restricted products (74 and 121 bp) represented the A/A genotype. To confirm the genotype found on PCR–RFLP approximately 50% of PCR sample products were randomly selected and subjected to direct sequencing using an ABI PRISM® 377 DNA sequencer.

IHC Study

Immunostaining of Bcl2 and Ki67 was performed on 56 formalin fixed, paraffin embedded renal cancer specimens. Anti –Bcl2 and anti-Ki67 antibodies (sc509, sc23900; Santa Cruz Biotechnology, Santa Cruz, CA) were used and the staining procedure was performed according to a commercial kit (Santa Cruz Biotechnology). The sections were counterstained with Harris hematoxylin. A pathologist evaluated the immunostaining while blinded. IHC staining was evaluated by assessing staining intensity on a scale of 0 to 3 using a microscope at 200×. All specimens were scored blindly by 2 observers. Expression intensity was scored as 0—negative, 1+—weakly positive, 2+—moderately positive and 3+—strongly positive with high expression considered a score of 2 and 3 (2 + 3/[0 + 1 + 2 + 3] × 100%).

Apoptosis and Proliferation

To detect apoptosis in renal cancer cells we used the TUNEL assay with an Apo-BrdU-IHC in situ DNA fragmentation assay kit (BioVision, Mountain View, California) according to manufacturer instruction. Apoptotic cell nuclei stained brown and nonapoptotic nuclei stained green. Apoptotic and total nuclei were counted in 10 fields at 100× magnification. The apoptotic index is expressed as the percent of apoptotic cells of the total number of cells in the given area. Ki-67 positive nuclei were counted in 10 fields at 100× magnification. The PI is expressed as the percent of Ki-67 positive cells of the total number of cells in the given area. Apoptosis and proliferation assessment was performed by a pathologist in blinded fashion.

Statistical Analysis

Hardy-Weinberg equilibrium was evaluated by SNPAlyze™, version 2.2 using an expectation maximization method. ANOVA and the chi-square test were used to compare clinical characteristics and genotype frequency between patients and controls. The OR was determined by unconditional logistic regression analysis and adjusted for age as a continuous variable. All statistical analyses were performed using StatView™, version 5 with $p < 0.01$ considered statistically significant. Genotype frequencies of the polymorphisms in 209 control and 216 case samples did not deviate from Hardy-Weinberg equilibrium ($p > 0.05$).

RESULTS

Patients With RCC and Controls

Characteristics. Table 1 shows mean age, gender, tumor grade and pathological findings in patients with RCC. The 2-tailed Student's t test and ANOVA were used to compare age and gender distributions between patients and controls. Of 216 RCC cases disease was localized in 163 (75.5%), grade 1 and 2 in 189 (87.5%), and the clear cell type in 203 (94.0%).

Genotype distribution and effect on clinical factors. Tables 2 and 3 list demographic and clinicopathological characteristics, including tumor grade and pathological TNM classification, in all RCC cases based on *bcl2* –938C/A genotypes. Genotype distribution and allele frequency in patients with RCC were not significantly different from those in controls (table 2). There was no positive correlation between *bcl2* −938C/A polymorphism and clinicopathological factors (table 2). Figure 1 shows a typical RFLP gel and sequencing.

Bcl-2 Expression and Genotype

To investigate the correlation between *bcl2* gene polymorphism (rs2279115) and Bcl2 protein expression IHC was done in 56 genotyped renal cancer tissues (fig. 2). Two of 56 formalin fixed, paraffin embedded renal cancer specimens were not clear cell carcinoma. Therefore, we assessed 54 clear cell carcinoma samples. We noted the expression level of Bcl2 in each genotype of the *bcl2* –938 C/A polymorphism. Figure 2, *A* shows the rate of Bcl2 expression in each genotype. The expression rate of AA, CA and CC genotypes was 80% (8 of 10 preparations), 52% (11 of 21) and 30% (7 of 23), respectively (fig. 2, *A*). Bcl2 expression was significantly lower in *bcl2* C/C genotype carriers (fig. 2, *A*). Bcl2 positive cells had brown stained cytoplasm. Figure 2, *B* shows representative Bcl2 expression.

Table 2. *Bcl2 genotype in patients with RCC and controls*

Bcl2-938C/A Genotype	No. Pts (%)	No. Controls (%)	p Value
Overall	216	209	
A/A	41 (19.0)	36 (17.2)	Referent
C/A	83 (38.4)	72 (34.4)	0.96
C/C	92 (42.6)	101 (48.3)	0.41
C/A+C/C	175 (81.0)	173 (82.8)	0.64

Table 3. *Demographic and clinicopathological characteristics, and bcl2 genotype in patients with RCC*

	No. Genotype				
	A/A	C/A	C/C	C/A + C/C	Total No.
Gender:					
F	13	31	23	54	67
M	28	52	69	121	149
p Value	Referent	0.53	0.42	0.92	
Grade:					
1 + 2	33	76	80	156	189
3 + 4	8	7	12	19	53
p Value	Referent	0.07	0.34	0.13	
pT:					
pT1 + pT2	30	68	65	133	163
pT3 + pT4	11	15	27	42	53
p Value	Referent	0.26	0.76	0.71	
pN:					
Neg	37	80	82	162	199
Pos	4	3	10	13	17
p Value	Referent	0.16	0.84	0.62	
pM:					
Neg	37	74	81	155	192
Pos	4	9	11	20	24
p Value	Referent	0.85	0.71	0.76	

IHC Results and Clinical Prognosis

Using Bcl2 IHC expression results overall survival was analyzed by the Kaplan-Meier method. Survival in Bcl2 positive cases was significantly longer than in negative cases (p = 0.0386, fig. 3).

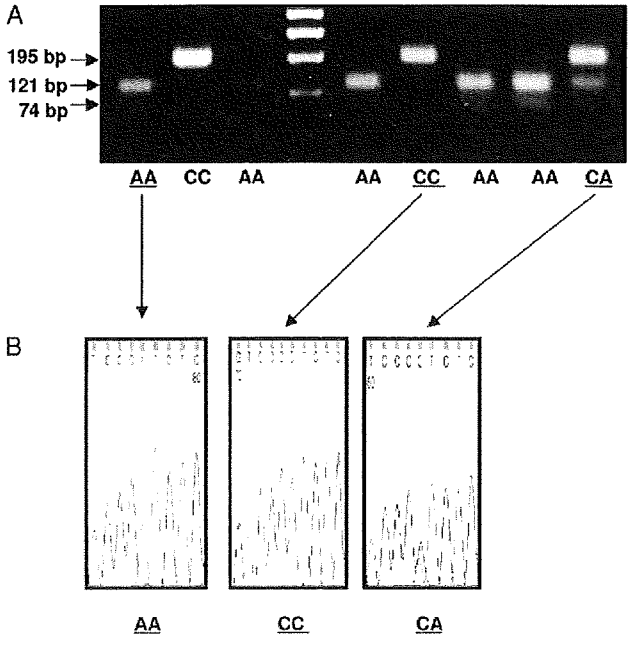

Figure 1. *A, bcl2* −938C/A polymorphism RFLP gels. Unrestricted 195 bp products represent C/C genotype, and restricted 74 and 121 bp products represent A/A genotype. *B,* DNA sequencing.

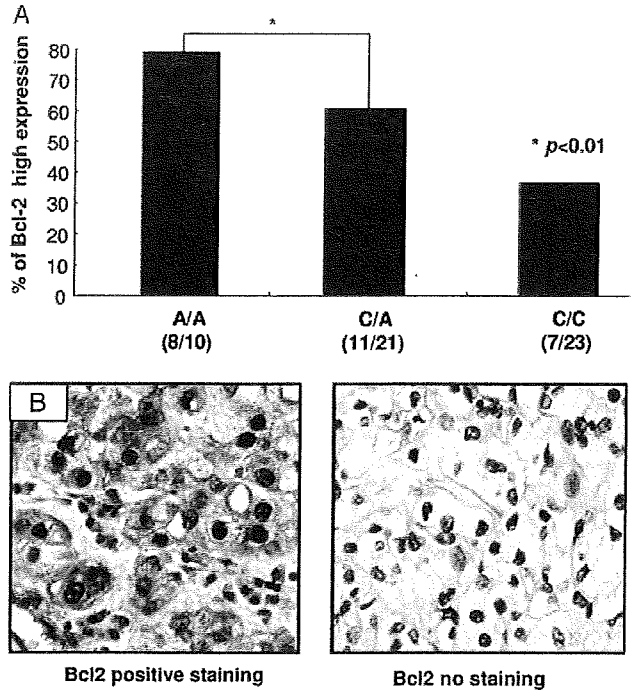

Figure 2. *A,* correlation between *bcl2* −938 C/A genotypes and Bcl2 expression. Asterisk indicates *bcl2* C/C vs other genotypes p value. *B,* representative Bcl-2 immunostaining in renal cancer. Reduced from ×200.

Apoptosis, Proliferation and *bcl2* Genotype

To investigate the correlation between *bcl2* gene polymorphism (rs2279115) and apoptosis the TUNEL assay was done in 54 genotyped renal cancer tissues. The apoptotic index was significantly higher in *bcl2* C/C carriers than in C/A and A/A carriers (median

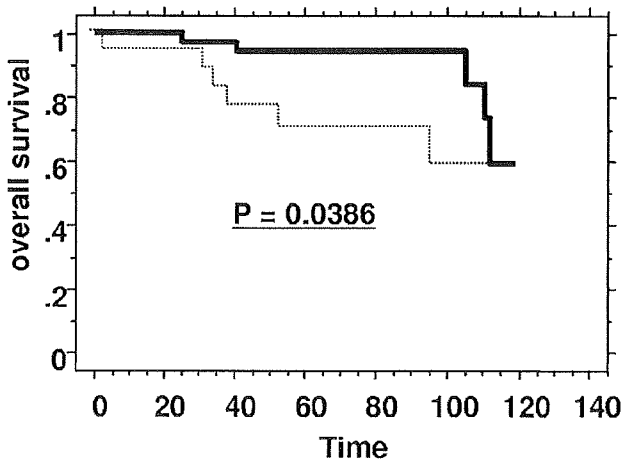

Figure 3. Overall survival based on Kaplan-Meier curves and Bcl2 expression on IHC in 56 patients with RCC. Bold line represents Bcl2 HIC positive in 38 patients. Dashed line indicates Bclt IHC negative in 18 patients.

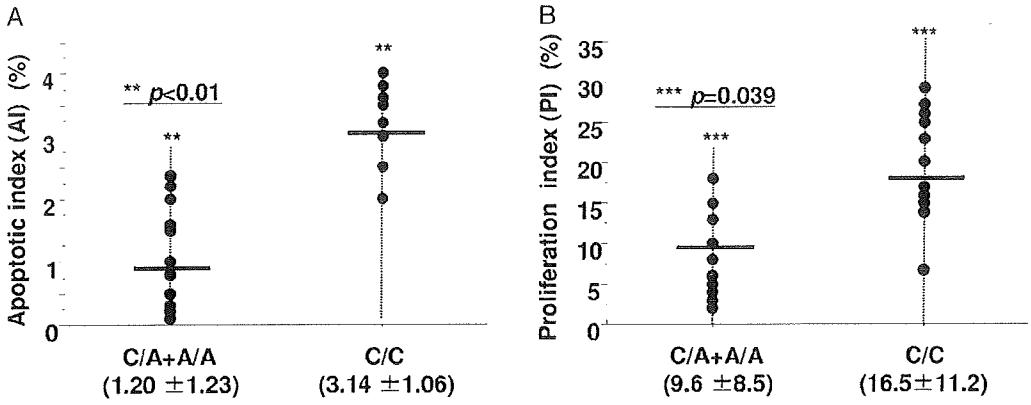

Figure 4. Genotyped RCC tissue *bcl2* −938C/A polymorphism. *A*, apoptosis. *B*, proliferation. Horizontal bars indicate mean. X axis indicates *bcl2* SNP genotype.

3.14 vs 1.20) (fig. 4, *A*). PI was also measured in the 54 genotyped RCC tissues. Similar to apoptosis results, PI was significantly higher in *bcl2* C/C carriers than in C/A or A/A carriers (median 16.5 vs 9.6) (fig. 4, *B*).

Cox Proportional Hazard Analysis of Overall Survival

The prognostic value of overall survival using parameters such as gender, age at diagnosis, pathological subtype, tumor grade, pTNM and *bcl2* SNP were analyzed using Cox proportional hazards analysis (table 4). On univariate analysis tumor grade, pT, pN, pM and *bcl2* SNP were associated with survival (table 4 and fig. 5). On multivariate analysis *bcl2* SNP and pTNM were independent risk factors for overall survival.

DISCUSSION

To our knowledge we report for the first time that the *bcl2 -938C/C* genotype is associated with a poor outcome in patients with RCC. On univariate analysis tumor grade, pTNM and *bcl2* SNP were statistically significant prognostic factors. In addition, multivariate Cox proportional hazard analysis revealed that this polymorphism could be an independent prognostic factor. Thus, we found a significant effect of the *bcl2* −938 C/A polymorphism on overall survival in patients with RCC.

Various groups have indicated the prognostic role of Bcl2 expression in various kinds of cancer and the effect of Bcl2 expression on prognosis is different among cancers.[6–14] In some cancers, such as breast, colon, kidney and nonsmall cell lung cancer, increased Bcl2 expression is associated with good prognosis, while there is an inverse association in other types.[6–14] In regard to RCC Itoi et al reported that Bcl2 expression significantly correlates with better survival.[14] Kallio et al reported that Bcl2 expression is associated with better prognosis in patients with RCC.[13] In our study there was a more favorable prognosis in Bcl2 positive vs negative cases, consistent with previous reports.[13,14]

To our knowledge there are no reports of the *bcl2* −938C/A polymorphism as a risk factor for renal cancer. Therefore, we analyzed the *bcl2* −938C/A polymorphism and Bcl2 expression in RCC tissues. We found that Bcl2 protein expression in RCC tissues was higher for the C/A+A/A genotype than for the C/C genotype. In patients with chronic lympho-

Table 4. *Univariate and multivariate Cox proportional hazard analysis of death risk in patients with RCC*

Parameter	Univariate		Multivariate	
	HR (95% CI)	p Value	HR (95% CI)	p Value
Gender (M vs F)	0.97 (0.43–2.18)	0.94		
Age at diagnosis (younger than 61 vs 61 or older)	1.84 (0.78–4.36)	0.16		
Pathological findings (clear cell Ca vs other)	1.61 (0.34–7.59)	0.54		
Grade (G2+G3+G4 vs G1)	2.3 (1.12–4.35)	0.03	1.4 (0.21–4.78)	0.77
pT (pT3+pT4 vs pT1+pT2)	4.87 (2.14–11.12)	0.0002	2.49 (1.25–5.05)	0.02
pN (pN1/pN2 vs pN0)	11.55 (2.24–59.53)	0.003	9.31 (1.36–63.65)	0.03
pM (pM1 vs pM0)	26.66 (5.50–129.12)	<0.0001	26.66 (4.40–129.21)	0.0002
Bcl2 SNP (rs2279115) (CC vs CA+AA)	2.4 (1.11–5.18)	0.026	2.16 (1.01–6.45)	0.048

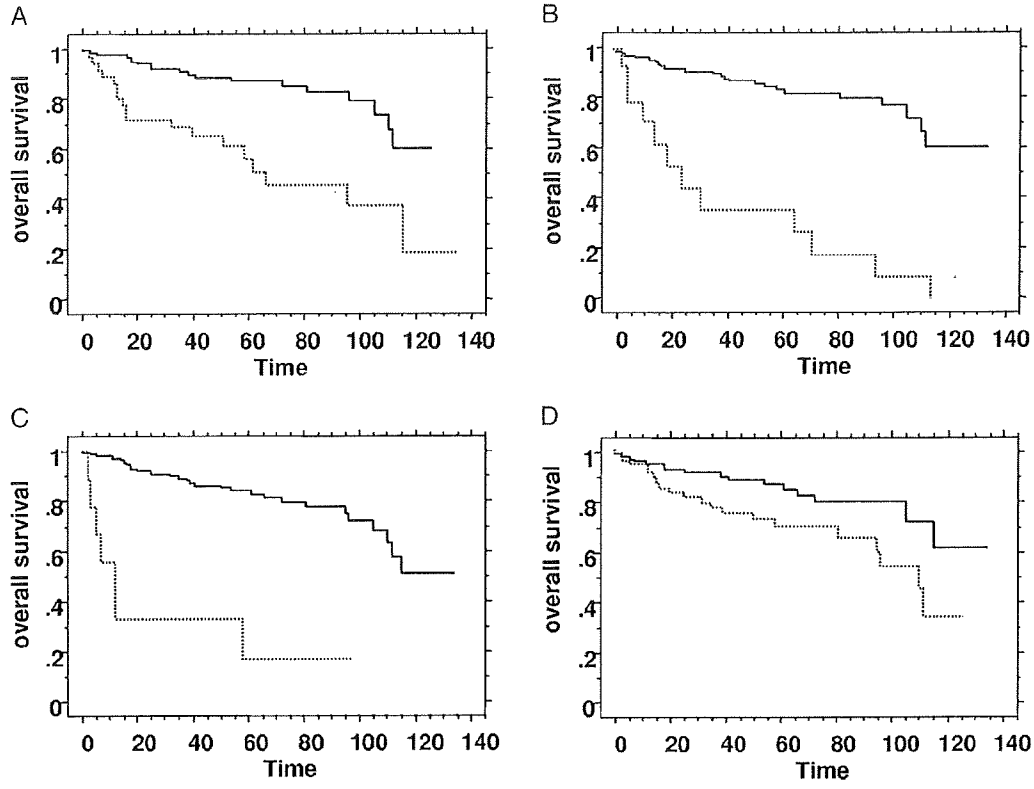

Figure 5. Overall survival based on Kaplan-Meier curves in 176 patients with RCC. *A*, tumor stage pT3/pT4 was independent predictor of overall survival ($p < 0.0001$). Solid line indicates pT1 and pT2. Dashed line indicates pT3 and pT4. *B*, distant metastasis was independent predictor of overall survival ($p < 0.0001$). Solid line indicates pM0. Dashed line indicates pM+. *C*, lymph node metastasis was independent predictor of overall survival ($p < 0.0001$). Solid line indicates pN0. Dashed line indicates pN+. *D*, *bcl2* C/C genotype was independent predictor of overall survival ($p = 0.0061$). Survival in C/C carriers (dashed line) was statistically significantly worse than in C/A+A/A carriers (solid line).

cytic leukemia and breast cancer the effect of the *bcl2* –938C/A polymorphism on survival is inconsistent, although Bcl2 protein expression in tissue from those with –938A/A genotypes was consistently increased compared with expression in those with the C/C genotype.[16,18] Our current results agree with these findings, namely that the *bcl2* –938A allele is tightly linked to increased Bcl2 expression.

The Bcl-2 family has an important role in apoptosis regulation and cell proliferation inhibition.[5] However, the data are mixed as to the relationship between Bcl2 expression and apoptosis or cell proliferation in cancer tissue. For instance, Sinicrope et al found that high Bcl2 protein levels significantly correlated with low proliferative activity in colon cancer cases.[19] However, others reported that Bcl2 expression is inversely associated with proliferative activity in nonHodgkin's lymphoma cases.[18] Similarly in RCC mixed results have been reported.[13,14,20] However, to our knowledge there has been no study to date of a correlation between the *bcl2* −938C/A polymorphism and RCC apoptosis or proliferation. Therefore, we compared apoptosis and cell proliferation with the *bcl2* –938C/A polymorphism using genotyped RCC tissue samples. These results show significantly higher TUNEL positive cells and proliferative cells in *bcl2* –938C/C carriers than in C/A+A/A carriers. These data suggest that the *bcl2* –938C/C genotype is associated with lower Bcl2 expression and higher cell proliferation in RCC cases. Previous reports have shown the relationship between Bcl2 expression and its inhibitory effect on cell proliferation in renal cancer, which supports our present data.[14,21] To our knowledge the molecular mechanisms of how Bcl2 is involved in carcinogenesis inhibition remain unknown. However, Bcl2 was described to be associated with cell cycle inhibition.[22] In an in vivo study increased Bcl2 expression delayed cell cycle entry and decreased cell proliferation.[22]

Since Bcl2 is an efficient inhibitor of drug induced apoptosis, it is important for renal cancer since almost no chemotherapy drugs are effective for RCC. Recently targeted tyrosine kinase inhibitors are used in advanced RCC cases[1] and numerous genes are thought to affect the success or failure of cancer chemotherapy.[23,24] Several mechanisms contribute

to chemotherapeutic drug resistance, such as DNA repair, apoptosis etc.[25] Bcl2 is a key contributor to apoptosis. Bachmann et al found that the *bcl2* −938C/A polymorphism is associated with prognosis in patients with breast cancer.[18] Our results agree with that study. Our current findings may shed new light on the mechanism behind this observation.

CONCLUSIONS

To our knowledge this is the first report of a significant effect of the *bcl2* −938C/A polymorphism on overall survival in patients with RCC. Furthermore, we noted that carriers with a *bcl2* promoter region −938C/A+A/A genotype have significantly higher Bcl2 expression and lower cell proliferative activity in RCC tissues than C/C genotype carriers. These data suggest that the *bcl2* −938C/A polymorphism is associated with survival in patients with RCC via altered Bcl2 expression.

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Expression of Autotaxin and Acylglycerol kinase in prostate cancer: Association with cancer development and progression

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Lysophosphatidic acid (LPA) may enhance diverse biologic activities in prostate cancer. This study was conducted to analyze expression levels of LPA-producing enzymes, autotaxin (ATX) and acylglycerol kinase (AGK), in prostate cancer with relevance to clinicopathological parameters. Real-time RT-PCR and western blotting were performed for ATX and AGK in non-neoplastic prostate cells (PrECs and PrSCs) and prostate cancer cell-lines (DU-145, PC-3, LNCaP, and AILNCaP). Immunohistochemical analyses were conducted in tissue specimens of 132 localized prostate cancer patients who underwent radical prostatectomy between 2001 and 2007 (median observation period, 22 months). Both enzymes were negatively expressed in PrECs and PrSCs at mRNA and protein levels. ATX expression was higher than AGK in AILNCaP, DU-145, and PC-3 cell-lines, while AGK was mainly expressed in LNCaP cells. Immunohistochemically, ATX and AGK expressions were negative in non-neoplastic epithelia, while both were weakly expressed in the majority of high-grade intra-epithelial neoplasia (HG-PIN). In cancer foci, ATX and AGK expressions were strong in 49% and 62%, weak in 40% and 32%, and negative in 11% and 6%, respectively. Expressions of both enzymes were significantly correlated with primary Gleason grade of cancer foci ($P < 0.0001$) and capsular invasion ($P = 0.03$ and 0.003 respectively). ATX expression was significantly correlated with probability of prostate specific antigen (PSA)-failure after surgery ($P < 0.0001$). In conclusion, LPA-producing enzymes (ATX and AGK) were frequently expressed in prostate cancer cells and precancerous HG-PIN. In particular, high expression levels of ATX were associated with both malignant potentials and poor outcomes. (*Cancer Sci* 2009; 100: 1631–1638)

Lysophosphatidic acid (1- or 2-acyl-lysophosphatidic acid; LPA) is an extracellular bioactive phospholipid that mediates diverse biological activities including platelet aggregation, smooth muscle contraction, cancer cell proliferation, invasion, angiogenesis, and cytoskeletal reorganization.[1,2] This action is mediated by several interactive mechanisms: (a) It activates *RhoA* and *NF-κβ* genes inducing prostate cancer progression.[3,4] (b) It enhances SRE activity in promoters of immediate early growth-related genes.[5] (c) It stimulates secretion of polypeptide growth factors such as EGF (epidermal growth factor) and sensitizes cells to their growth promoting effects.[6,7] (d) Finally, LPA suppresses apoptosis of cancer cells by reducing levels of apoptosis-promoting proteins.[8,9]

We previously examined LPA activity in various biologic fluids and found a high LPA activity exerted by a specific type of its receptors (Edg-7/LPA3) in human seminal fluids.[10] Furthermore, addition of 18:1 LPA (oleoyl-LPA) to prostate epithelial and stromal cells resulted in up-regulation of a novel extracellular matrix signaling protein CYR-61, that has a growth stimulating potential.[11]

Several routes are proposed for LPA production. It is produced extracellularly by lipoprotein oxidation through the action of secretory phospholipase A2 on microvesicles released from activated cells.[12] In plasma, it is produced by thrombin-activated platelets through the stimulated release of phospholipase-A1 and A2[13] and lysophospholipase D (LysoPLD).[14,15] LysoPLD is identical to autotaxin (nucleotide pyrophosphatase phosphodiesterase-2; ATX/NPP2, EC 3.1.1.5), a cell motility-stimulating factor originally identified in the culture cell supernatant of malignant melanoma cells.[16,17] We previously found that human seminal fluids contain a large amount of ATX, which hydrolyses lysophosphatidylcholine to produce LPA.[18] While LPA signaling had been extensively investigated over the past decade, researchers are now beginning to appreciate the properties and biological activities of ATX as the major LPA-producing phospholipase.[19] ATX is a secreted protein that acts outside the cell to produce LPA. It was recently suggested that ATX activity increases LPA production which is believed to promote prostate cancer development and progression via a pro-inflammatory milieu.[20] Another potential pathway for LPA synthesis is phosphorylation of monoacylglycerols by a specific lipid kinase, AGK (EC 2.7.1.94).[1,21] Regarding LPA metabolism, we carried out biochemical characterization of ejaculate from healthy males and found that PAP, a legend clinical marker for prostate cancer, is responsible for degrading LPA.[22]

These findings have prompted us to investigate the expression levels of LPA-producing enzymes, ATX and AGK, in prostate cancers and their association with different clinical behaviors and outcomes. In this study, expressions of ATX and AGK in prostate cancer cell-lines were analyzed at both mRNA and protein levels with a special relevance to androgen-sensitivity. Furthermore, surgical specimens were examined for ATX and AGK protein expressions in cancer foci, HG-PIN, and non-neoplastic glands to clarify the relationship of ATX and AGK expressions to clinicopathological features of prostate cancer.

Materials and Methods

Cell lines. Prostate epithelial (PrECs) and stromal (PrSCs) cells were purchased from Bio Whittaker (Walkersville, MD, USA) and maintained in the specific basal media according to the manufacturer's recommendation. Human prostate cancer cell-lines DU-145, PC-3 and LNCaP were purchased from the American Type Culture Collection (Rockville, MD, USA) and

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Table 1. Patients' clinicopathological characteristics

Characteristics	Study cohort (n = 132)
Follow-up (months)	
Mean	26.2
Median	22
Age (years)	
<60	10 (7.6%)
60–70	53 (40.2%)
>70	69 (52.2%)
Pretreatment PSA (ng/mL)	
<4	2 (1.5%)
4–10	61 (46.2%)
10–20	53 (40.2%)
>20	16 (12.1%)
Pathological stage (TNM 1997)	
≤pT2a	17 (12.8%)
pT2b	59 (44.7%)
pT3a	37 (28.0%)
≥pT3b	19 (14.5%)
Pathological Gleason score	
≤3 + 3	13 (9.8%)
3 + 4	39 (29.5%)
4 + 3	36 (27.3%)
≥4 + 4	44 (33.4%)
Surgical margin	
Negative	88 (66.7%)
Positive	44 (33.3%)

PSA, prostate-specific antigen.

were maintained in RPMI-1640 plus 10% FCS (Life Technologies, NY, USA). Androgen-insensitive LNCaP subline (AILNCaP) was proliferated in androgen-depleted medium after emerging from long-term androgen-depleted cultures of the androgen-sensitive LNCaP cells as previously described.[23]

Patients and specimens. Whole prostate specimens were obtained from 132 patients with localized prostate cancer (cT1c-2bN0M0, International Union Against Cancer [UICC] 1997) who underwent radical prostatectomy at Kagawa University Hospital during the period between January 2001 and December 2007 (median observation period, 22 months). The patients' characteristics are shown in Table 1. None of our patients received preoperative or adjuvant hormonal ablation therapy, chemotherapy, or radiotherapy until PSA-failure was confirmed. Written informed consent was obtained from all participants. This study complies with the Declaration of Helsinki in 1995 (as revised in Tokyo, 2004) and was approved by the institutional ethical committee.

RNA extraction and real-time RT-PCR. To elucidate ATX and AGK gene expression profiles in prostate cancer cell-lines, RNA was isolated using RNAqueous-Micro RNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA samples were treated with DNase using the Invitrogen DNase I (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized as described elsewhere.[24] AGK forward and reverse primers have been described elsewhere.[1] ATX amplification primers were as follows: forward, GGGTGAAAGCTGGAACA TTCTT; reverse, GAAGGCCTCTCATGATCTGG. Real-time RT-PCR was conducted on a LightCycler system (Roche Diagnostics, Indianapolis, IN, USA). Reaction mixture in 20 μL was amplified for 40 cycles using: 95°C for 10 s, 65–67°C for 10 s, and 72°C for 15 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was determined with a ready-to-use LightCycler-h-GAPDH housekeeping gene set (Roche Applied Science, Indianapolis, IN, USA) to confirm integrity. Fluorescence data were analyzed with reference to the standard curve in each experiment. A melting curve analysis was always included to certify amplification. Target gene expression was referred to GAPDH mRNA for normalization. All tests were conducted in triplicate to ensure reproducibility. The specificity of desired products in real-time RT-PCR was also confirmed by conventional RT-PCR using the same primer pairs as previously described.[25]

Antibodies. Anti-ATX rat monoclonal antibody (clone 4F1) was established in the laboratory of the Graduate School of Pharmaceutical Sciences, University of Tokyo, as previously described.[22] Anti-AGK rabbit polyclonal antibody (OB0680) was newly developed at MBL (Nagoya, Japan) from sera of rabbits immunized with a synthetic peptide. The amino acid sequence of that peptide is CDPRKREQMLTSPTQ, corresponding to codon 408 to 422 of the C-terminal region of AGK. The specificity of OB0680 was confirmed by a blocking experiment in which immunostaining was carried out for representative specimens using either a mixture solution of the synthetic peptide described above and OB0680 or OB0680 alone as the primary antibody. The positive staining in cancer foci reacted with OB0680 alone disappeared when a mixture solution was used as the primary antibody.

H_2O_2-streptavidin biotinylated antirat IgG (for ATX) or antirabbit IgG (for AGK) were used as secondary antibodies (Dako, Glostrup, Denmark).

Protein extraction and western blot analyses. At the exponential growing phase, PrECs and PrSCs, as well as DU-145, PC-3, and LNCaP cells, were lysed and protein extracts were prepared. Cell proteins were electrophoretically separated and transferred to PVDF membranes (Immobilon; Daiichi Pure Chemicals, Tokyo, Japan). Proteins were analyzed with 1:1000 dilutions of anti-ATX or anti-AGK antibodies. Secondary antibodies were then added and horseradish peroxidase (HRP)–conjugated streptavidin (American Pharmacia, Piscataway, NJ, USA) was used in conjunction with an ECL chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) for assessing protein expression. To control for protein loading, membranes were re-probed with mouse monoclonal β-actin antibodies (AC-15, ab6276-100; Abcam, Cambridge, MA, USA).

Tissue preparation and immunostaining. Surgical specimens were immediately fixed in 10% buffered neutral formalin and embedded in paraffin. Five microgram–thick sections were prepared from tissue blocks and subjected to hematoxylin–eosin staining. The overall Gleason score and the primary Gleason grade of major cancer foci in each tumor were determined by the institutional pathologist (R.H.). Sections harboring major cancer foci were selected for further ATX and AGK immunostaining using the standard immunoperoxidase immunohistochemistry as previously described.[26] Briefly, after deparaffinization with xylene and rehydration with graded ethanol, antigen retrieval was accomplished using 10-mM sodium citrate buffer (pH 6) being heated in a microwave at 500 W. Endogenous avidin and biotin were suppressed using an avidin/biotin blocking agent (Nichirei, Tokyo, Japan). Non-specific protein interactions were blocked by incubation with 1–2% blocking serum (normal rabbit serum in PBS; Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight at 4°C with 1:40 dilution of anti-ATX antibody or 1:1000 dilution of anti-AGK antibody. The bound antibodies were visualized by the streptavidin–biotin method including HRP and diaminobenzidine (DAB) chromogen according to manufacturer's instructions (ABC kit; Vector Laboratories).

Assessment of staining intensity. Prior to this study, immunohistochemistry for ATX and AGK was carried out as a preliminary setting using tissue specimens obtained from 20 randomly selected radical prostatectomy patients. This preliminary immunostaining revealed that both ATX and AGK were negative in non-neoplastic glands and stroma without exception,

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whereas HG-PIN lesions were usually positive for both enzymes although the staining intensity was weak. In contrast, ATX and AGK were strongly positive in cancer foci in several different prostate specimens. As an inter-experimental validation, one representative specimen was selected from the preliminary study for assessment of ATX and AGK expression as a referring control. These reference specimens consisted of cancer foci with strong protein expression, high-grade PIN lesions with weak expression, and normal glands with negative staining. Assessment of staining intensity was carried out only when immunostaining of the reference specimens showed comparable results. Based on the above mentioned parameters, we determined the grade of staining intensity in the present study as follows: grade 0 was completely negative (similar to non-neoplastic glands); grade 1 was weakly positive (similar to the majority of HG-PIN lesions); and grade 2 was of a staining intensity much stronger than that of HG-PIN with positive expression.

Statistical analysis. All statistics were performed with the Statistical Package for Social Sciences software, version 16 (SPSS, Chicago, IL, USA). The statistical results are reproducible and differences found are not due to random variation. Clinicopathological parameters were compared by χ^2 analysis and Fischer's exact test. Postoperative PSA-failure-free survival was determined using the Kaplan–Meier method and the log-rank test was used to compare the resultant curves. PSA failure was defined as a PSA level at or above 0.2 ng/mL following surgery.[27] For examining the relationship between ATX and AGK expression level and the pathological T-stage, our patients were classified into two groups: (T2b or less) and (T3a or more) according to the 1997 TNM staging system. For multivariate analyses, the Cox proportional hazards model was used. Statistical significance of differences was set at $P < 0.05$.

Results

Autotaxin (ATX) and AGK expressions in prostate cancer cells. Expression profiles of LPA-producing enzymes ATX and AGK were quite different between androgen-insensitive DU-145, PC-3, and AILNCaP (the androgen-insensitive subline of LNCaP) cells and androgen-sensitive LNCaP cells at both mRNA and protein levels (Fig. 1). Quantitative assessment of ATX mRNA in prostate cancer cell lines is shown in Figure 1a. ATX mRNA level in DU-145 was much higher than LNCaP ($P = 0.0027$) and higher than PC-3 cells ($P = 0.0028$), which was comparable to protein expression levels confirmed by western blotting (Fig. 1b). Moreover, the ATX mRNA level in AILNCaP cells was significantly higher than that in parental LNCaP cells ($P = 0.0028$). On the other hand, the AGK mRNA level (Fig. 1c) in LNCaP cells was significantly higher than that of PC-3 and DU-145 ($P < 0.0001$ and $P = 0.0005$ respectively), which was comparable in western blot analyses (Fig. 1d). The AGK mRNA level was significantly lower in AILNCaP cells as compared with its parental LNCaP cells ($P = 0.0167$). On the other hand, protein expression levels of both enzymes in non-neoplastic cells (PrECs and PrSCs) were very weak or negative.

Autotaxin (ATX) and AGK protein expressions in prostate cancer tissue specimens. In benign glands and their surrounding stroma, both ATX and AGK were immunohistochemically negative for all specimens examined (Figs 2a and 3a). HG-PIN lesions were identified adjacent to major cancer foci in 109 (82.5%) out of 132 patients. ATX was weakly positive in 83 (76.1%) cases (Fig. 2b) and AGK was positive in 91 (83.5%) out of the 109 HG-PIN lesions (Fig. 3b). Consequently, both enzymes were weakly but simultaneously expressed in HG-PIN lesions obtained from 83 out of 109 patients (Table 2a).

In contrast, major cancer foci in 65 (49%) patients showed strong expression of ATX (a representative picture is shown in Fig. 2d) while those in 53 (40%) patients showed weak expression (Fig. 2c) and the remaining 14 (11%) showed negative expression. Major cancer foci in 82 (62%) patients showed strong expression of AGK (representative pictures are shown in Fig. 3d,e), while those in 42 (32%) patients showed weak expression (Fig. 3c) and the remaining eight (6%) cases showed negative expression. The positive staining in cancer foci was not identified by the addition of the synthetic peptide, CDPRKREQMLTSPTQ, to the anti-AGK antibody (Fig. 3f), which indicates specific reactivity of the antibody to cancer epithelia. Consequently, major cancer foci obtained from 45 (34%) patients showed strong expression of both ATX and AGK while staining intensities of both enzymes did not coincide in more than half of the subjects (Table 2b).

Table 2a. A comparison between ATX and AGK expression in HG-PIN lesions*

AGK expression (n = 109/132, 82.5%)	ATX expression (n = 109/132; 82.5%)	
	Grade 0 (n = 26; 23.9%)	Grade 1 (n = 83; 76.1%)
Grade 0 (n = 18; 16.5%)	18	0
Grade 1 (n = 91; 83.5%)	8	83

*Significant correlation $P < 0.0001$. AGK, acylglycerol kinase; ATX, autotaxin; HG-PIN, high-grade prostate intraepithelial neoplasia.

Table 2b. A comparison between ATX and AGK expression in cancer foci*

AGK expression	ATX expression		
	Grade 0 (n = 14)	Grade 1 (n = 53)	Grade 2 (n = 65)
Grade 0 (n = 8)	3	4	1
Grade 1 (n = 42)	5	18	19
Grade 2 (n = 82)	6	31	45

*Insignificant correlation $P = 0.199$. AGK, acylglycerol kinase; ATX, autotaxin.

As to the cellular localization of ATX and AGK expressions in cancer foci, both were positive in the cytoplasm. In addition, nuclear staining of ATX was observed in the cancer foci of 40 (30.3%) patients (Fig. 2d). Moreover, ATX nuclear staining was more frequently observed in higher Gleason grades (4 or 5) than in lower (3 or less) grades ($P = 0.0002$).

Staining intensities and clinicopathological parameters. Table 3 shows the relationship of ATX and AGK expression levels with various clinical and pathological parameters in our prostate cancer patients. Both ATX and AGK expression levels were significantly correlated with primary Gleason grade of major cancer foci that were immunohistochemically analyzed ($P < 0.0001$) as shown in Figures 2 and 3. Moreover, presence of capsular invasion was correlated with strong expression of ATX ($P = 0.03$) and AGK ($P = 0.003$). On the contrary, pathologic T-stage, perineural infiltration, seminal vesicle invasion, and lymphatic or vascular spread were not correlated with ATX or AGK expression levels.

Prostate-specific antigen (PSA)-failure and expression levels of ATX and AGK. Figure 4 shows the relationship between biochemical failure-free survival and staining intensities of ATX and AGK. Probability of PSA-progression was significantly correlated with ATX staining intensity ($P < 0.0001$) but not with AGK staining intensity. A multivariate analysis including ATX and AGK staining intensities, preoperative serum PSA level, Gleason score, pathologic T-stage, and other clinicopathological parameters demonstrated that ATX staining intensity ($P < 0.0001$), lymphatic involvement ($P = 0.041$), and serum PSA levels ($P = 0.022$) were independent prognostic markers for PSA-progression (Table 4).

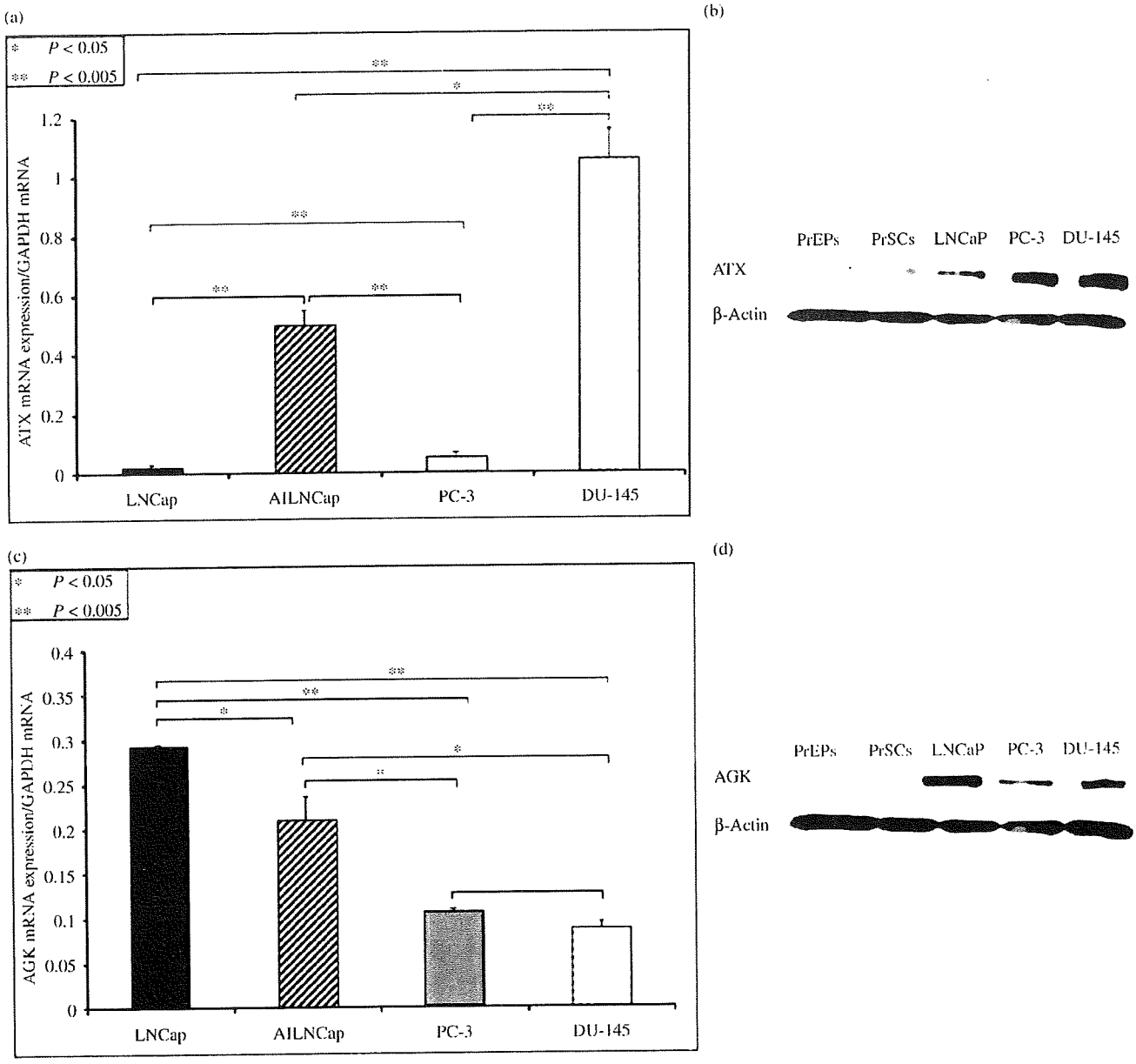

Fig. 1. Expression profile of lysophosphatidic acid (LPA)-producing enzymes autotaxin (ATX) and acylglycerol kinase (AGK), both at mRNA (real-time RT-PCR relative to GAPDH) and protein (western blot analysis relative to β-actin) levels in non-neoplastic prostate cells (PrECs, PrSCs) and prostate cancer cell lines. (a) ATX mRNA level is significantly higher in androgen-insensitive prostate cancer cell lines (DU-145, AILNCaP, and PC-3) than in the androgen-sensitive LNCaP cells. (b) ATX protein expression is significantly higher in androgen-insensitive prostate cancer cell lines (DU-145 and PC-3) than in the androgen-sensitive LNCaP cells, while it is very weakly expressed in non-neoplastic cells (PrECs, PrSCs). (c) AGK mRNA level is significantly higher in the androgen-sensitive LNCaP cells than in androgen-insensitive prostate cancer cell lines (DU-145, AILNCaP, and PC-3). (d) AGK protein expression is significantly higher in the androgen-sensitive LNCaP cells than in androgen-insensitive prostate cancer cell lines (DU-145 and PC-3), while it is negative or very weakly expressed in non-neoplastic cells (PrECs, PrSCs). Error bars represent the SEM for data from four experiments.

Discussion

Our study is the first to analyze expression levels of LPA-producing enzymes ATX and AGK in prostate cancer with a special relevance to its clinicopathological profiles in order to clarify the role of LPA in the development and progression of prostate cancer.

Autotaxin (ATX) and AGK were significantly expressed in prostate cancer tissues while the surrounding non-neoplastic glands were negative in all specimens studied. Weak but apparent expression of LPA-producing enzymes in HG-PIN, in contrast to negative expression in normal glands, suggests that LPA-producing enzymes or LPA itself play a key role in the development of prostate cancer. As to the relationship of both enzymes' expression to malignant potentials of prostate cancers, the primary Gleason grade of cancer foci and presence of capsular invasion were significantly correlated with expression levels of ATX and AGK. These findings suggest that LPA-producing

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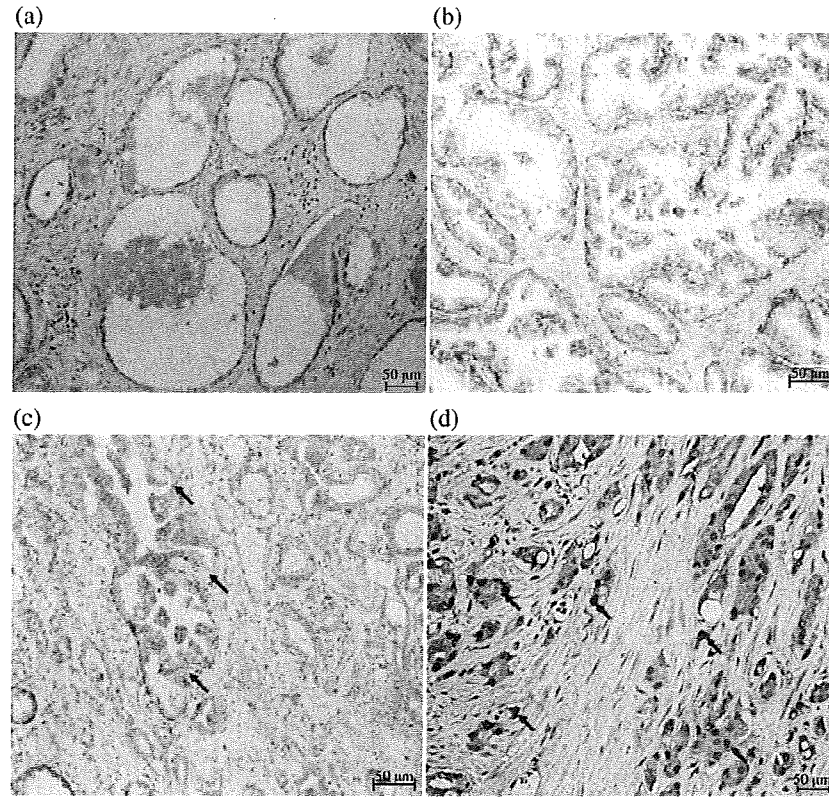

Fig. 2. Immunohistochemical staining of autotaxin (ATX) in prostate cancer specimens with different Gleason grades. (a) Grade 0 expression in non-neoplastic prostatic tissues. (b) Grade 1 expression in a high-grade prostate intraepithelial neoplasia (HG-PIN) lesion. (c) Grade 1 expression in Gleason grade 3 cancer foci similar to the staining intensity of the nearby HG-PIN lesions (black arrows). (d) Grade 2 expression in a Gleason grade 5 lesion with a characteristic nuclear staining in cancer cells (black arrows). The magnification bar measures 50 μm.

Table 3. Staining intensity of ATX and AGK in comparison with clinicopathological profiles of prostate cancer patients

Parameters		ATX expression			*P*-values	AGK expression			*P*-values
		Grade 0 ($n = 14$)	Grade 1 ($n = 53$)	Grade 2 ($n = 65$)		Grade 0 ($n = 8$)	Grade 1 ($n = 42$)	Grade 2 ($n = 82$)	
Gleason score	≤3 + 3	0	10	3	0.16	0	7	6	0.05
	3 + 4	4	16	19		4	15	20	
	4 + 3	4	13	19		2	13	21	
	≥4 + 4	6	14	24		2	7	35	
Primary Gl. grade	≤Gl. 3	11	38	12	<0.0001	6	31	24	<0.0001
	≥Gl. 4	3	15	53		2	11	58	
Pathologic T-stage	≤T2b	6	34	36	0.32	6	28	42	0.15
	≥T3a	8	19	29		2	14	40	
Capsular invasion	Negative	7	39	33	0.03	5	34	40	0.003
	Positive	7	14	32		3	8	42	
Perineural invasion	Negative	4	13	11	0.47	2	12	14	0.32
	Positive	10	40	54		6	30	68	
SV invasion	Negative		44	49	0.27	7	34	65	0.85
	Positive	1	9	16		1	8	17	
Lymphatic involvement	Negative	9	30	30	0.34	6	22	41	0.40
	Positive	5	23	35		2	20	41	
Vascular involvement	Negative	11	35	43	0.64	7	32	50	0.11
	Positive	3	18	22		1	10	32	
Mean PSA (ng/mL)		12.1	15.2	11.9	0.36	11.9	14.5	13	0.41

Note: Significant values are underlined.
AGK, Acylglycerol kinase; ATX, Autotaxin; Gl., Gleason; PSA, prostate-specific antigen; SV, seminal vesicle.

enzymes or LPA also play a key role in the progression of prostate cancer. Our recent analyses using laser capture microdissection showed that Edg-7/LPA3, a LPA-receptor, was over-expressed in prostate cancer and its expression level was significantly correlated with the primary Gleason grade of cancer foci examined.[28] Furthermore, the previous study revealed that protein localization of ATX and AGK was restricted to cancer epithelial cells. The aforementioned studies,[1,6,9,10,28] together with the present results, suggest that the LPA-rich microenvironment is advantageous for the malignant progression of prostate cancers.

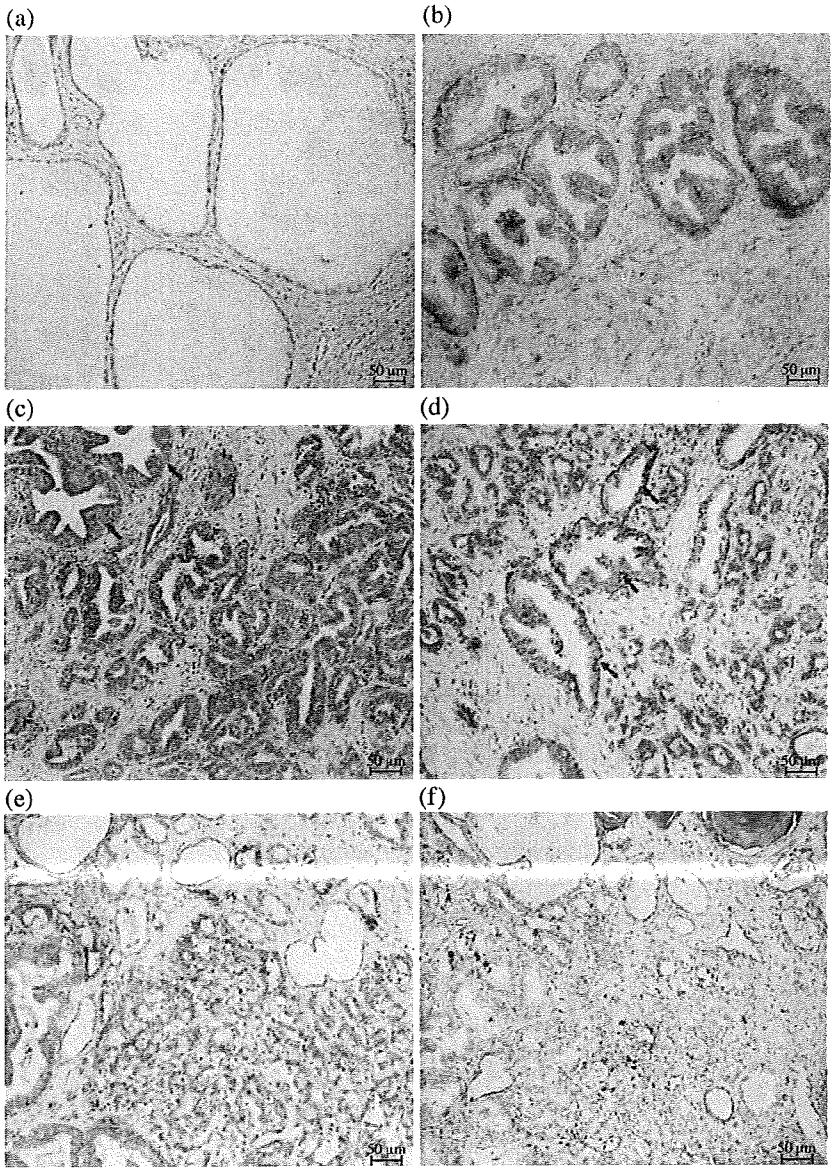

Fig. 3. Immunohistochemical staining of acylglycerol kinase (AGK) in prostate cancer specimens with different Gleason grades. (a) Grade 0 expression in non-neoplastic prostatic tissues. (b) Grade 1 expression in a high-grade prostate intraepithelial neoplasia (HG-PIN) lesion. (c) Grade 1 expression in Gleason grade 3 cancer foci (similar staining intensity to HG-PIN lesions). (d) Grade 2 expression in cancer foci of Gleason grade 4. Notice the characteristic difference in staining intensity between the strongly stained cancer foci and the weakly stained nearby HG-PIN lesions (black arrows). (e) Another grade 4 cancer foci with grade 2 expression. (f) The strong staining disappeared when the synthetic peptide CDPRKREQMLTSPTQ was mixed with the anti-AGK antibody OB0680. The magnification bar measures 50 μm.

Autotaxin (ATX) and AGK expression patterns were quite different between prostate cancer cell-lines as well as tissue specimens. Altered expression profiles were encountered at both mRNA and protein levels in prostate cancer cell-lines in which ATX was mainly expressed in the androgen-insensitive DU-145 and PC-3 cells, while AGK was more predominant in the androgen-sensitive LNCaP cells. Moreover, in the androgen-insensitive subline of LNCaP cells (AILNCaP), AGK expression was low as compared to their androgen-sensitive counterparts. In contrast to that, ATX mRNA level was higher in AILNCaP than in LNCaP cells. These results are comparable to the immunohistochemical findings in surgical specimens in which staining intensity of ATX did not always coincide with that of AGK. Rather, 37 (28%) tumors with strong AGK staining intensity showed weak or negative staining for ATX, while 20 (15%) tumors with strong ATX staining intensity showed weak or negative staining for AGK. Furthermore, AGK expression was solely limited to the cytoplasm while ATX was significantly expressed in the nuclei of cancer cells as well. This difference was particularly noticed in higher Gleason grade cancer foci with high ATX expression. The difference in subcellular localization between ATX and AGK can be ascribed to the potential nuclear role played by ATX, while AGK is mainly localized in the mitochondria.[4,5,29] These altered expression patterns can be explained by both enzymes not acting simultaneously in LPA synthesis in which phosphorylation could be the dominant pathway, particularly during early stages of prostate oncogenesis. Previous studies also showed that ablation of endogenous AGK markedly reduced LPA-dependent EGF activity and cell proliferation.[1] Others have postulated that ATX is inhibited in a negative feedback fashion by LPA which is not the case in AGK.[30] Interestingly, ATX mRNA level in PC3 cells was lower than that in DU-145 in all experiments done including our previous study,[28] but ATX mRNA could always be identified. It remains to be elucidated whether post-translational modification plays a role in the protein expression of ATX in PC3 cells.

In the present study, ATX expression was inversely correlated to the probability of PSA-failure-free survival, which was particularly interesting because ATX has been reported to be a cell motility–stimulating factor.[16] Our results showed that ATX

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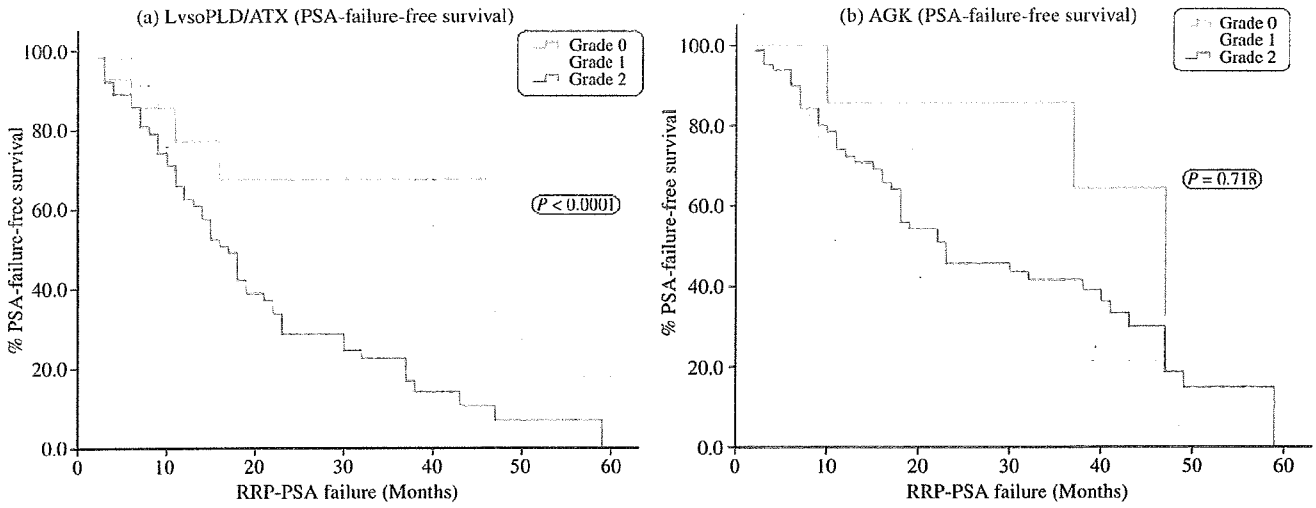

Fig. 4. Prostatespecific antigen (PSA)-failure-free survival curves in patients divided into three groups according to the staining intensity of autotaxin (ATX) expression levels (a) or acylglycerol kinase (AGK) expression levels (b). Staining intensity of ATX was significantly correlated with probability of PSA-failure ($P < 0.0001$) whereas that of AGK was not significant ($P = 0.718$). LysoPLD, lysophospholipase D; RRP, Radical Retropublic Prostatectomy.

Table 4. Multivariate Cox regression analysis of ATX and AGK expression patterns and clinicopathological profiles of prostate cancer in relation to the PSA-failure-free survival outcome in our patients

Parameter	Variable	RR	95% CI	*P*-values
ATX expression	Grade 0 *vs* Grade 1	2.679	1.216–5.906	<u><0.0001</u>
	Grade 1 *vs* Grade 2	1.045	0.543–2.011	
	Grade 0 & 1 *vs* Grade 2	1.927	1.360–2.732	
AGK expression	Grade 0 *vs* Grade 1	1.470	0.601–3.600	0.109
	Grade 1 *vs* Grade 2	0.578	0.319–1.047	
	Grade 0 & 1 *vs* Grade 2	0.764	0.549–1.062	
PSA level	≤(4) *vs* (4–10)	0.483	0.103–2.255	<u>0.022</u>
	(4–10) *vs* (10–20)	4.226	1.282–13.93	
	(10–20) *vs* ≥(20)	0.897	0.473–1.704	
Gleason score	≤(3 + 3) *vs* (3 + 4)	1.176	0.562–2.458	0.299
	(3 + 4) *vs* (4 + 3)	0.648	0.348–1.209	
	(4 + 3) *vs* ≥(4 + 4)	1.489	0.912–2.431	
pT-stage	≤(T2b) *vs* ≥(T3a)	1.444	0.787–2.649	0.235
Capsular invasion	Negative *vs* Positive	0.676	0.369–1.237	0.204
Perineural invasion	Negative *vs* Positive	0.846	0.509–1.405	0.518
SV invasion	Negative *vs* Positive	1.257	0.786–2.011	0.339
Lymphatic involvement	Negative *vs* Positive	1.574	1.018–2.433	<u>0.041</u>
Vascular involvement	Negative *vs* Positive	0.719	0.469–1.103	0.130
Surgical margin	Negative *vs* Positive	1.331	0.949–1.896	0.098

Note: Significant values are underlined.
AGK, acylglycerol kinase; ATX, autotaxin; CI, confidence interval; PSA, prostate-specific antigen; pT-stage, pathologic T-stage; RR, relative risk; SV, seminal vesicle.

could be an independent prognostic tissue marker for predicting PSA failure outcome in prostate cancer patients. However, it remains to be elucidated in a prospective fashion whether ATX is a potential marker for prediction of outcome after treatment intervention for localized prostate cancer,[31] as it may provide a promising target in therapies of prostate cancer.[17]

In conclusion, although concentration of LPA in prostate cancer foci was not directly evaluated, LPA-producing enzymes (ATX and AGK) were frequently expressed in cancer cells and precancerous HG-PIN. In particular, high expression levels of ATX were correlated with both malignant potentials and poor outcomes. These results strongly suggest that an ATX–(or AGK–)LPA axis plays a pivotal role in both the development and progression of prostate cancer.

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Disclosure

No potential conflict of interest could be perceived as prejudicing the impartiality of research.

Abbreviations

AGK	Acylglycerol kinase
ATX	Autotaxin
HG-PIN	High-grade prostate intraepithelial neoplasia
LPA	Lysophosphatidic acid
NF-κB	Nuclear factor–kappa β gene
PAP	Prostatic acid phosphatase
PSA	Prostate-specific antigen.
RhoA	Ras homolog A gene
SRE	Serum response element

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Original Article: Clinical Investigation

Quality of life after radical prostatectomy in elderly men

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Objectives: To evaluate the impact of radical prostatectomy (RP) on health-related quality of life (HRQOL) in elderly men with prostate cancer.

Methods: Between January 2002 and December 2006, a total of 205 elderly men (≥70 years) undergoing RP participated in our longitudinal outcome study. Patients completed general (Short Form 36) and disease-specific (University of California, Los Angeles Prostate Cancer Index) HRQOL questionnaires. A t-test was used to compare each HRQOL score after RP with the baseline scores and Cox proportional hazard models to characterize the recovery trends.

Results: Patients undergoing RP showed physical problems, which diminished over time. Several emotional domains significantly improved during the follow-up period. By 2 years postoperatively, 57% and 81% of subjects had fully returned to baseline urinary function and bother, respectively. Mean recovery time to baseline urinary function and bother was 8.3 months and 4.7 months, respectively. When incontinence was defined as 'no pad', 82% of patients reported continence. Whereas only 25% of patients returned to the baseline sexual function level, 83% had reached baseline sexual bother. Among those returning to their own baseline scores, the mean recovery time was 10.9 months for sexual function and 5.3 months for sexual bother.

Conclusions: Selected elderly patients can achieve satisfactory functional outcomes after RP. These outcomes should be considered when decisions about treatment are made.

Key words: elderly men, prostate cancer, quality of life, radical prostatectomy.

Introduction

Decision-making for patients with prostate cancer over the age of 70 years is becoming a major area of debate because patients are living longer than in the past. In a previous study, the Japanese subjects with prostate cancer tended to be older than the American subjects.[1] Life expectancy calculations in the USA suggest an upper age limit of 70–72 years for those offered radical prostatectomy (RP). Currently, the life expectancy of a 75-year-old Japanese man is 11.2 years,[2] while the life expectancy of a 75-year-old American man is 10.3 years.[3]

It is not uncommon these days for urologists to be faced with a healthy patient over 70 years old and newly diagnosed with localized prostate cancer. The 10-year life expectancy requirement governs the decision-making for physicians faced with strong evidence of organ-confined disease in an elderly patient.[4] In particular, the role of RP among elderly men remains controversial above the age of 70. Elderly men with early stages of cancer often live long after the diagnosis and desire to maximize their health-related quality of life (HRQOL).[4] Many studies have addressed the effects of treatments for prostate cancer on HRQOL outcomes, but little has been published on QOL following RP in men over the age of 70. In the present study, we address one aspect of this debate by looking at HRQOL after RP.

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Methods

Patient population and data collection

Between January 2002 and December 2006, a total of 653 patients who underwent RP participated in our longitudinal outcome study at Tohoku University Hospital and its affiliated hospital. Among these patients, 205 RP patients (31%) were 70 years old or older and were included in this analysis. All recruitment and research protocols were approved by the Ethics Committee, Tohoku University School of Medicine. All patients were informed of their cancer diagnosis before being asked to fill out the HRQOL questionnaires. Those who agreed to participate in the present study received from their urologist a questionnaire, an informed consent form, and a prepaid envelope for returning the questionnaire. The baseline interview was conducted before the initiation of treatment. Follow-up assessments were completed 3, 6, 12, 18, and 24 months after RP.

QOL methodology and statistical analysis

We evaluated general HRQOL with the RAND 36-Item Short Form Health Survey (SF-36).[5,6] The general scales cover eight domains, four physical and four emotional. The prostate-specific HRQOL was assessed with the University of California, Los Angeles Prostate Cancer Index (UCLA-PCI).[7] The UCLA-PCI encompasses urinary, bowel, and sexual problems and the extent of bother from problems in each areas. The SF-36 and UCLA-PCI quality of life scores for the various domains are shown as mean ± standard deviation (SD) in scales of 0 to 100, with a higher score always representing a better HRQOL. Both questionnaires have been translated into Japanese and the validity and reliability were previously tested.[8,9]

Treatment protocols

All of the staff urologists carried out RP and used virtually the same technique originally described by Walsh.[10] Every surgeon had considerable experience with the retropubic approach before the beginning of the study. The indications for a treatment option depended on preoperative factors such as the needle biopsy pathology parameters of Gleason score, along with the clinical parameters of serum prostate-specific antigen (PSA) and digital rectal examination.[11]

Statistical analysis

The analysis focused on comparing each HRQOL score of the postoperative groups with the baseline scores. For these comparisons, we a conducted *t*-test and used a *P*-value of less than 0.05 to denote statistical significant. In addition, we focused on several definitions with regard to urinary continence to assess the return to the individual baseline level postoperatively. For this part of the analysis a patient was considered to have achieved continence only when the follow-up score returned to the baseline level. Next, we assessed urinary and sexual function and bother using the principles of survival analysis with Cox proportional hazard models to characterize the recovery trends. This outcome of measuring a patient's returning to pretreatment values is a clinically meaningful change.[12] We created models based on the occurrence of each subject's return to his own baseline score. A subject was considered to have returned to baseline if his domain score was at least 90% of his baseline. Once a subject returned to baseline, his time to return was censored.

Results

Background characteristics of the study group

Of these 205 subjects, seven patients who received neoadjuvant therapy were excluded because it was considered to have possibly affected the recovery of HRQOL. Only patients with preoperative HRQOL data and data from at least two later times were included in the analysis, resulting in a final study cohort of 195 who underwent RP.

Table 1 lists the selected demographic and clinical characteristics of the study sample. The mean patient age was 72.5 years (range 70 to 78). The respondents showed a median preoperative PSA of 12.6 ng/mL (range 3.3–54.0). Histopathologically organ-confined disease was found in 79% ($n = 155$) of the surgical specimens. Most patients (77%) experienced comorbidities, the most common of which were hypertension (29%), diabetes (20%), gastrointestinal (18%), cardiovascular (12%) disease, and other kinds of carcinoma (7%), but these comorbidities were well controlled. Of the 195 subjects, 78 (40%) patients did not undergo nerve preservation, and 117 (60%) patients underwent either unilateral (87 [74%] patients) or bilateral (30 [26%] patients) nerve-sparing surgery. Twenty-eight (14%) subjects received salvage therapy during the study period. At the time of the baseline survey, 94% of the men were married or lived with a partner and 52% were employed.

HRQOL assessment

The questionnaire submission rates among these patients were 100%, 90%, 91%, 95%, 83%, and 85% at baseline, 3, 6, 12, 18, and 24 months after treatment, respectively. A longitudinal assessment of the general and cancer-specific HRQOL scores is also shown in Tables 2 and 3, respectively. We compared the age-adjusted scores for the eight domains of SF-36 with the reference population of the Japanese population. All domains scores of the SF-36 were comparable to those of the reference population. Of the eight SF-36 domains, role limitations due to physical problems significantly decreased at 3 months ($P = 0.033$), but at 6 months these domains recovered to the baseline. Role limitations due to emotional problems and vitality in postoperative periods were statistically higher than baseline. Mental health scores were statistically higher after one year postoperatively. Other domains showed no significant difference between baseline and any of the observation periods. There were no significant differences in any of the general HRQOL domains between the subjects who received postoperative salvage therapy and those who underwent RP alone (data not shown).

According to UCLA-PCI scores that represent disease-specific HRQOL, the urinary function substantially declined at 3 months and continued to recover at 6, 12, 18, and 24 months but scored lower than the baseline ($P < 0.01$). Urinary bother had a significantly worse score at 3 and 6 months than that at baseline ($P < 0.01$). At 12 months after surgery, however, it returned to the baseline. In the domain of bowel function and bother, no significant difference was observed between the baseline and any of the post-treatment

Table 1 Demographic and clinical characteristics of study population

No. patients	195
Age (years)	
Mean (SD)	72.5 (1.9)
75 or less	164
More than 75	31
PSA at diagnosis (ng/mL)	
Mean (SD)	12.6 (16.6)
10 or less	131
More than 10	64
Pretreatment tumor stage	
T1	97
T2	75
T3	23
Pathological tumor stage	
T0	1
T2	154
T3	40
Gleason score	
7 or less	116
More than 7	79
Nerve-sparing procedure	
Bilateral	30
Unilateral	87
None	78
Salvage therapy	
None	167
Radiation therapy	7
LH–RH analog	6
Antiandrogen	10
LH–RH analog plus antiandrogen	2
Radiation therapy plus hormonal therapy	3
Cormobidity count	
0	44
1	90
2	43
3 or more	18
Working status	
Full-time worker	51
Part-time worker	9
Retired/no job	135
Marital or relationship status	
Married or living with spouse or partner	177
Unmarried or not in significant relationship	18

LH–RH, luteinizing hormone-releasing hormone; PSA, prostate-specific antigen; SD, standard deviation.

times. The score for sexual function declined over the 24 months. Similarly, sexual bother scored significantly lower at each postoperative time point ($P < 0.05$).

Baseline levels of urinary continence measured by preoperative questionnaire responses varied according to the definition of continence applied. Definition 1 (greater than 'very small problems' with leaking urine) was a sensitive definition of urinary incontinence reported by 5.2% ($n = 10$). According to definition 2 ('leakage more than once a day') and 3 ('no pads') 13% and 3% of patients, respectively, were categorized as incontinent at baseline. According to definition 2, 39% of the patients remained incontinent at 24 months postoperatively. On the other hand, when continence was defined according to definition 3, overall 43%, 65%, 71%, 77%, and 82% of men were continent at the 3, 6, 12, 18, and 24-month follow up points, respectively. Moreover, most of the subjects (84%) felt very small or no problem with leaking urine at 24 months after RP based on definition 1 (Fig. 1).

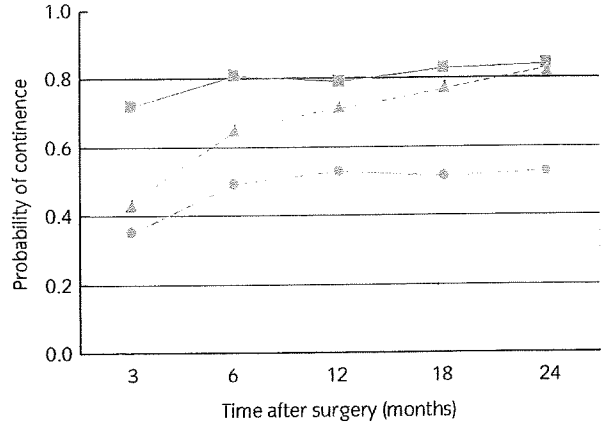

Fig. 1 Longitudinal analysis of patient probability of continence following radical prostatectomy (RP) stratified by definition 1 ('very small' or 'no problem' with urinary leakage), definition 2 (leakage frequency less than 'once per day') and definition 3 ('no pad'). ■, definition 1; ●, definition 2; ▲, definition 3.

Kaplan–Meier curves were made to represent the return to the baseline HRQOL score. By 2 years postoperatively, the proportion of those returning to the baseline urinary function and bother level was 57% and 81%, respectively. The mean recovery time of urinary function and bother was 8.3 months and 4.7 months, respectively. On the other hand, recovery of sexual function and bother to the baseline level was 25% and 83%, respectively, at 24 months after RP. The mean recovery time of sexual function and bother was 10.9 months and 5.3 months, respectively. (Fig. 2)

Discussion

The current study has several important findings. First, the RP subjects in our cohort had favorable functional outcomes in spite of their representing an elderly population. Specifically, in several domains of SF-36, those who underwent RP had significant declines in role limitation due to physical

Table 2 SF-36 scores of patients treated with radical prostatectomy

Physical function		*P*-value	Mental health		*P*-value
Baseline	85 ± 12		Baseline	79 ± 21	
3 M	84 ± 12		3 M	74 ± 19	
6 M	84 ± 14		6 M	80 ± 19	
12 M	84 ± 12		12 M	83 ± 19	*
18 M	86 ± 12		18 M	86 ± 18	*
24 M	84 ± 15		24 M	84 ± 19	*
Role limitation due to physical problems			Bodily pain		
Baseline	79 ± 21		Baseline	69 ± 20	
3 M	73 ± 25	*	3 M	66 ± 19	
6 M	78 ± 23		6 M	70 ± 21	
12 M	80 ± 20		12 M	69 ± 19	
18 M	82 ± 21		18 M	72 ± 20	
24 M	83 ± 21		24 M	70 ± 19	
Role limitation due to emotional problems			Vitality		
Baseline	83 ± 22		Baseline	70 ± 18	
3 M	80 ± 24		3 M	74 ± 19	
6 M	82 ± 22		6 M	75 ± 18	
12 M	86 ± 22		12 M	76 ± 18	
18 M	87 ± 23		18 M	78 ± 20	*
24 M	88 ± 22	*	24 M	74 ± 19	
Social function			General health		
Baseline	83 ± 22		Baseline	58 ± 14	
3 M	79 ± 21		3 M	60 ± 15	
6 M	82 ± 20		6 M	60 ± 18	
12 M	83 ± 18		12 M	60 ± 17	
18 M	83 ± 20		18 M	62 ± 16	
24 M	80 ± 18		24 M	61 ± 17	

Statistically significant changes from baseline are indicated as *$P < 0.05$. Data are presented as mean ± standard deviation. SF-36, RAND 36-Item Short Form Health Survey.

problems, but had significant improvements in these scores up to 6 months, with values almost returning to the pretreatment levels. Radical surgery appears to offer an effective treatment option for elderly men and should be considered in certain subsets of patients. Selection of the treatment depends on the health status, such as comorbidities as well as the stage of disease or age. The results of the current study confirmed the findings of earlier studies which showed that healthy, elderly subjects and those with no or mild comorbidity tended to undergo RP.[13,14] In general, RP is thought to be beneficial for patients with an estimated life-expectancy of >15 years.[15] Age has a strong influence on the treatment pattern; younger men prefer RP, middle-aged men prefer external beam radiation therapy (EBRT) and older men prefer either no treatment or hormonal therapy.[14] In our series, 31% of the RP subjects in our cohort were 70 years or older in part because many Japanese urologists consider 75 years as the upper age limit for RP. Krahn *et al.* found that the odds of a 75-year-old man being offered RP for a moderately differentiated tumor were only 0.003 times those of a 55-year-old man being offered the same treatment.[16] Suzuki *et al.* reported that elderly Japanese men with prostate cancer present with similar histologic grade and disease stage compared with younger men.[17] Physicians and elderly patients should consider these outcomes in making a decision about the treatment for prostate cancer.

Second, for our subjects at 24 months following RP, the rate of recovery of urinary function and bother score was 57% and 81%, respectively. Urinary incontinence is a concern particularly relevant to men undergoing RP because surgery more frequently negatively affects continence than other treatment modalities, and because patients rate urinary status as one of their greatest concerns regarding HRQOL. It has been shown that age is associated with a greater likelihood of returning to baseline continence, potency and physical health after RP.[18] We compared these results with those from another large study that included 247 RP patients.[12] In the study by Litwin *et al.*, 56% and 71% recovered baseline

Table 3 UCLA-PCI of patients treated with radical prostatectomy

Urinary function		*P*-value	Urinary bother		*P*-value
Baseline	92 ± 14		Baseline	87 ± 24	
3 M	62 ± 24	**	3 M	71 ± 27	**
6 M	72 ± 21	**	6 M	79 ± 23	*
12 M	75 ± 18	**	12 M	82 ± 16	
18 M	78 ± 19	**	18 M	82 ± 20	
24 M	79 ± 19	**	24 M	84 ± 20	
Sexual function			Sexual bother		
Baseline	24 ± 24		Baseline	67 ± 28	
3 M	6 ± 13	**	3 M	60 ± 35	*
6 M	7 ± 13	**	6 M	57 ± 34	*
12 M	8 ± 16	**	12 M	60 ± 34	*
18 M	9 ± 16	**	18 M	57 ± 34	*
24 M	9 ± 16	**	24 M	57 ± 31	*
Bowel function			Bowel bother		
Baseline	88 ± 15		Baseline	91 ± 15	
3 M	83 ± 19		3 M	88 ± 21	
6 M	85 ± 15		6 M	88 ± 18	
12 M	84 ± 16		12 M	88 ± 20	
18 M	86 ± 15		18 M	90 ± 18	
24 M	87 ± 14		24 M	89 ± 17	

Statistically significant changes from baseline are indicated as $*P < 0.05$ and $**P < 0.01$, respectively. Data are presented as mean ± standard deviation. UCLA-PCI, University of California, Los Angeles Prostate Cancer Index.

urinary function and bother scores, respectively, which was comparable with our current study. Moreover, in our previous studies, urinary function is significantly better in younger patients than elderly patients immediately after RP, but both groups appear similar by the end of year 2.[19] Thus, when a well-selected patient over the age of 70 chooses surgery, the urinary QOL can be expected to be not too different from that found in studies looking at all ages.

Third, using a self-reported questionnaire, the elderly subjects in this age cohort reported low sexual function before and after treatment. These findings are consistent with other reports that Japanese men reported less sexual activity than did American men, but Japanese men were less likely to be bothered.[20] Sexuality is a complex entity with physical, emotional, psychological, cultural, and religious dimensions that differ from person to person. Interestingly, in this dataset, although there was a fairly strong correlation between urinary function and bother ($r = 0.58$), there was a much weaker correlation between sexual function and bother ($r = 0.18$). This disconnection between sexual function and bother illustrates the importance of an individual's perception of a disease state and the age-dependent differences in the impact of the loss of sexual function. The pattern of help-seeking behavior varies widely among countries. In East Asian countries including Japan, most people took no action, while in South-east Asian countries help was more often sought from partners, families, or other social supports.[21] Japanese elderly men may view 'iatrogenic sexual dysfunction' as not only a normative side effect of cancer treatment but also a natural part of aging.

We acknowledge several limitations in this prospective observational study. First, because there was no randomization of the treatment, the results might not be representative of all elderly patients receiving RP, and there is potential for inherited treatment bias. Second, the sample was relatively small and may not be representative of the general elderly population. In addition, we did not use a sample of younger patients for comparison, which is likely to have introduced bias. Third, we did not distinguish among those who used erectile aids such as type 5 phosphodiesterase inhibitors or vacuum devices and there was no documentation of use of alpha-blockers or anticholinergics. These factors may be significant predictors of urinary or sexual function recovery. Finally, trends in HRQOL might differ for such individuals. A selection bias may also have occurred with regard to patients who agreed to participate in this study.

Despite these limitations, our findings must be confirmed or refuted by the longitudinal data of others. A richer understanding of the changes in HRQOL after RP will enable physicians to provide clinically relevant information that allows elderly patients who elect RP to be comfortable

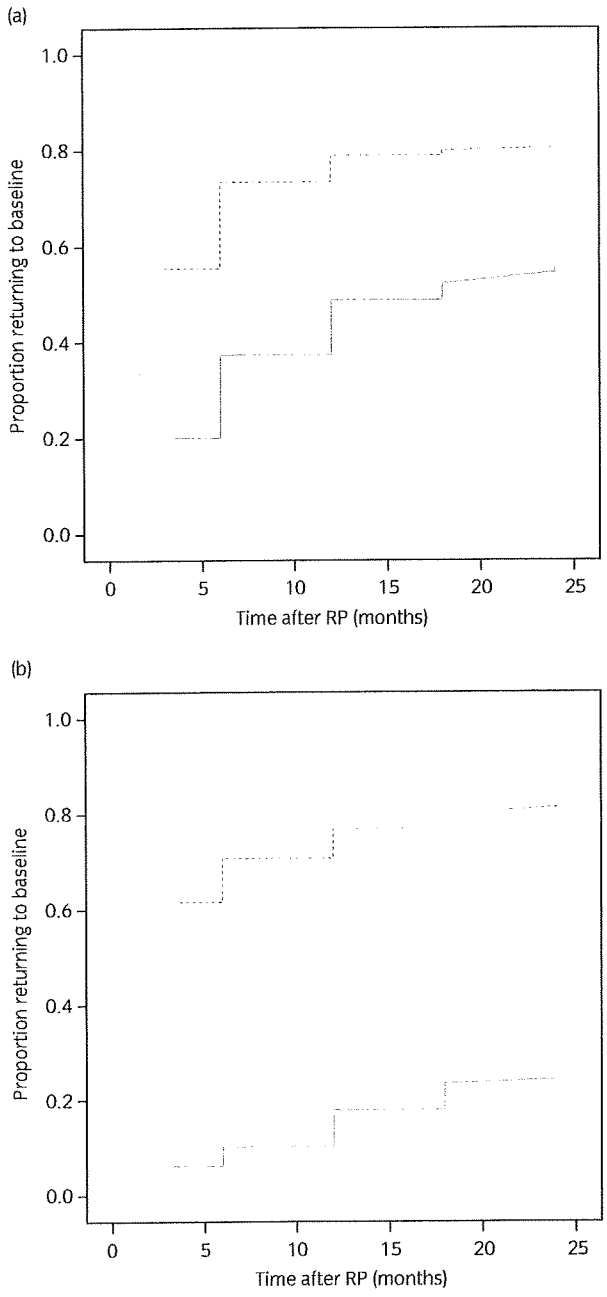

Fig. 2 Kaplan–Meier analysis of the proportion of radical prostatectomy (RP) subjects returning to baseline score over time. (a) Urinary function (solid line) and bother (dashed line). (b) Sexual function (solid line) and bother (dashed line).

with their choices. The emphasis on chronologic age should be shifted to 'biologic age' when elderly men are diagnosed with localized prostate cancer. Physicians should continue their concerted efforts to help patients make informed treatment decisions based not only on survival predictions but also on health status, functional concerns, and most importantly, personal preference.

Elderly patients appear to have a better tolerance for RP from the HRQOL aspect. Healthy elderly patients with localized prostate cancer should not be limited in their treatment options solely on the basis of advanced chronologic age. Thus, managing prostate cancer in this group requires a comprehensive assessment and multidisciplinary approach to maximize their HRQOL.

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