Table 1. Outcomes of histologic examination and real-time RT-PCR assay

	Group A	Group B	Group C
No. of patients	11	32	77
No. of dissected lymph nodes	201	619	1,395
Histologic examination			
No. of positive patients	11	0	0
No. of positive lymph nodes	29	0	0
Real-time RT-PCR assay for PSA			
No. of positive patients	11	23	0
No. of positive lymph nodes	51	84	0
Real-time RT-PCR assay for PSMA			
No. of positive patients	11	29	0
No. of positive lymph nodes	71	112	0
Micrometastasis			
No. of positive patients	11	32	0
No. of positive lymph nodes	82	143	0

NOTE: Group A, patients with histologically confirmed lymph node metastases; group B, patients with micrometastases despite the lack of histologic evidence indicating nodal involvement; and group C, patients without any findings of lymph node metastases.

(Abacus Concepts, Inc., Berkeley, CA), and $P < 0.05$ was considered significant.

Results

The expression of GAPDH mRNA in all lymph node specimens was confirmed. In 148 lymph nodes from seven female patients with bladder cancer, the mean values of relative PSA and PSMA mRNAs expression plus 2 SDs were 2.8 and 4.9, respectively, and these values were used as cutoff points for the positive expression of PSA and PSMA mRNA in lymph nodes from patients with prostate cancer in the subsequent study. Real-time RT-PCR assays in 2,215 pelvic lymph nodes from patients with clinically localized prostate cancer detected various amounts of relative expression levels of PSA and PSMA mRNAs (PSA: mean, 2.5; median, 0.6; range, 0-193; PSMA: mean, 4.4; median, 1.2; range, 0-792).

Twenty-nine of the 201 lymph nodes from 11 patients with prostate cancer showed histopathologic evidence of metastatic involvement, and real-time RT-PCR confirmed the expression of PSA and PSMA mRNAs in 29 and 28 nodes, respectively. In these 11 patients, positive PSA and/or PSMA mRNA expression was detected in an additional 53 histologically uninvolved lymph nodes; thus, a total of 82 lymph nodes were diagnosed as having occult micrometastases using real-time RT-PCR assay. Of the 2,014 nodes from the remaining 109 patients without histologic evidence of pelvic lymph node metastases, positive PSA and PSMA mRNA expression were detected in 84 nodes from 23 patients and 112 nodes from 29 patients, respectively. Among these, 53 nodes from 20 patients were judged positive for both PSA and PSMA mRNAs expression; therefore, a total of 32 patients were regarded as having micrometastases to pelvic lymph nodes. The relative expression levels of PSA and PSMA mRNAs in 225 nodes considered positive for micrometastases were as follows: (PSA) mean, 17.2; median, 15.1; range 1.9 to 193; (PSMA) mean, 32.4; median, 24.4; range, 3.1 to 792. These outcomes are summarized in Table 1 by dividing 120 patients into the following three groups: 11 with histologically detected lymph node metastases (group A), 32 with micrometastases despite the lack of histologic evidence indicating nodal involvement (group B), and the remaining 77 without any findings of lymph node metastases on histologic and real-time RT-PCR analyses (group C).

The incidence of micrometastases according to anatomic location was analyzed. Similar metastatic patterns of prostate cancer cells to the external iliac region and obturator fossa were observed between groups A and B, irrespective of the presence of histologically confirmed nodal involvement. We further compared clinicopathologic features among these three groups. As shown in Table 2, despite the absence of significant differences between groups A and B in several of the factors examined, preoperative serum PSA, pathologic stage, Gleason score, and tumor volume in groups A and B were significantly greater than those in group C.

The median follow-up period of the 120 patients included in this study was 38 months (range, 15-48 months). In this series, biochemical recurrence occurred in 8, 17, and 7 patients in groups A, B, and C, respectively (Table 3). The median intervals between radical prostatectomy and biochemical recurrence in groups A, B, and C were 6, 11, and 17 months, respectively. As

Table 2. Comparison of conventional prognostic indicators according to lymph node metastases detected by histologic examination and real-time RT-PCR assay

	Groups			*P*		
	A	B	C	A vs. B	B vs. C	C vs. A
No. of patients	11	32	77			
Preoperative serum PSA (ng/mL)*	25.3 ± 24.7	20.2 ± 18.7	9.8 ± 6.9	0.48	<0.0001	<0.0001
Pathologic stage (no. of patients)				0.23	0.0008	<0.0001
pT_2	1	11	55			
pT_3	9	20	22			
pT_4	1	1	0			
Gleason score*	8.1 ± 4.1	7.5 ± 3.9	6.1 ± 2.9	0.67	0.041	0.046
Tumor volume (cm^3)*	2.5 ± 1.7	2.0 ± 1.4	0.92 ± 0.65	0.34	<0.0001	<0.0001

NOTE: Group A, patients with histologically confirmed lymph node metastases; group B, patients with micrometastases despite the lack of histologic evidence indicating nodal involvement; group C, patients without any findings of lymph node metastases.
*Data are presented as mean ± SD.

Table 3. Incidence of biochemical recurrence according to lymph node metastases detected by histologic examination and real-time RT-PCR assay

	Groups			*P*		
	A	B	C	A vs. B	B vs. C	C vs. A
No. of patients	11	32	77			
No. of patients with biochemical recurrence (%)	8 (72.7)	17 (53.1)	7 (9.1)	0.26	<0.0001	<0.0001
Mean time to biochemical recurrence after radical prostatectomy (mo)*	9.5 ± 9.9	14.9 ± 7.6	21.0 ± 9.9	0.86	0.032	0.0004
Pathologically organ-confined disease						
No. of patients with biochemical recurrence/total no. of patients	0/0	4/11	2/55	—	0.0006	—
Pathologically extraprostatic disease						
No. of patients with biochemical recurrence/total no. of patients	8/11	13/21	5/22	0.54	0.092	0.0056

NOTE: Group A, patients with histologically confirmed lymph node metastases; group B, patients with micrometastases despite the lack of histologic evidence indicating nodal involvement; group C, patients without any findings of lymph node metastases.
*Data are presented as mean ± SD.

shown in Fig. 1, biochemical recurrence-free survival rates in groups A and B were significantly lower than that in group C. However, there was no significant association between the number of positive nodes for micrometastases as well as quantitative values of PSA and PSMA expression with biochemical recurrence (data not shown). In addition, of the 66 patients with pathologically organ-confined disease, only 6 developed biochemical recurrence, among whom 4 were diagnosed as having micrometastases in the pelvic lymph nodes (Table 3).

To evaluate the association between several clinicopathologic factors with biochemical recurrence, multivariate analysis using a stepwise logistic regression model was done. As shown in Table 4, only the presence of micrometastasis was independently related to whether or not biochemical recurrence occurred. Furthermore, multivariate analysis using the Cox regression hazard model showed that only the presence of micrometastasis was independently associated with biochemical recurrence-free survival, irrespective of other factors examined in this study (Table 4).

To further confirm the presence of micrometastatic diseases in pelvic lymph nodes, immunohistochemical stainings were done with a monoclonal antibody against PSA in 143 lymph nodes from 32 patients diagnosed as having micrometastases using real-time RT-PCR assays (despite the lack of pathologic evidence of nodal involvement). Sixty-one of the 143 lymph nodes (from 17 patients) were evidently stained with PSA antibody. Representative results are shown in Fig. 2.

Discussion

Lymph node metastasis is the most useful factor predicting poor prognosis in patients undergoing radical prostatectomy for clinically localized prostate cancer. However, ~30% of such patients without evidence of pathologic nodal involvement will develop biochemical disease recurrence (1, 2). Although the etiology of biochemical disease recurrence following radical prostatectomy is likely multifactorial, a significant proportion of these recurrences might be due to occult metastases to pelvic lymph nodes undetected by routine pathologic examinations. Several investigators have assessed whether microscopic foci of prostate cancer cells are present in histologically uninvolved pelvic nodes using molecular and histochemical approaches (3-6), but the clinical significance of micrometastases in pelvic nodes remains controversial. Because accurate staging of prostate cancer facilitates the prediction of therapeutic outcomes and appropriate tailoring of adjuvant therapies to the individual patient, we investigated PSA and PSMA mRNA expression in 2,215 pelvic lymph nodes dissected at radical prostatectomy from 120 patients with clinically localized prostate cancer using quantitative real-time RT-PCR assay, evaluated the sensitivity of this assay for detecting occult lymph

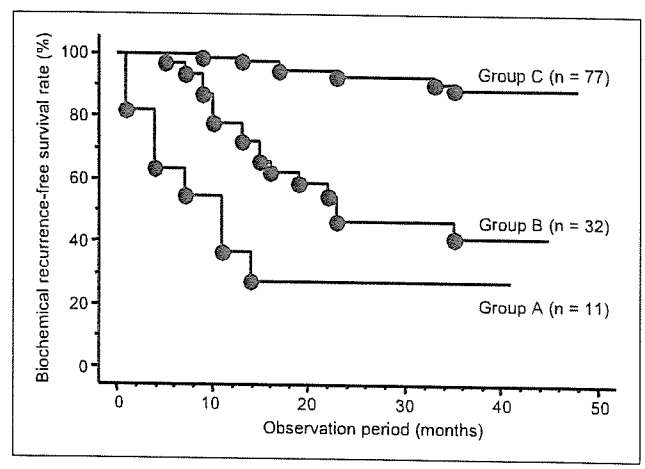

Fig. 1. Comparison of biochemical recurrence – free survival rates in groups A, B, and C using the Kaplan-Meier method. The biochemical recurrence – free survival rates in groups A and B were significantly lower than that in group C ($P = 0.059$, group A versus group B; $P < 0.0001$, group B versus group C; $P < 0.0001$, group C versus group A using the log-rank test).

Table 4. Multivariate analyses of various factors in relation to biochemical recurrence

Variables	*P* value	
	Stepwise logistic regression model	Cox proportional hazards regression model
Serum PSA, ng/mL (<10 vs. ≥10)	0.55	0.61
Pathologic stage (pT_2 vs. pT_3 or pT_4)	0.23	0.51
Gleason score (6 or 7 vs. 8, 9, or 10)	0.14	0.10
Tumor volume, cm^3 (<1.0 vs. ≥1.0)	0.44	0.33
Micrometastasis (negative vs. positive)	0.040	0.032

node metastases, and analyzed various clinicopathologic factors according to the assay findings.

In this series, standard pelvic lymphadenectomy targeting the external iliac region and obturator fossa for all 120 patients was done, and the mean number of lymph nodes removed at radical prostatectomy in these patients was 18.5. Based on an autopsy study, approximately 20 lymph nodes have been shown to serve as a guideline for optimal and representative pelvic lymph node dissection (17), suggesting that the procedure for pelvic lymphadenectomy done in this study, which met this requirement, would be suitable. We also examined 148 pelvic lymph nodes obtained from seven female patients undergoing radical cystectomy for invasive bladder cancer to determine the appropriate cutoff points for the positive expression of PSA and PSMA mRNAs on real-time RT-PCR. Although it is a potentially crucial point to reduce the false positivity of real-time RT-PCR, it is usually difficult to establish cutoff points on this assay for diseases lacking specific markers. However, PSA and PSMA gene expressions are highly restricted to prostate epithelial cells (4); that is, although it is inevitable to detect extremely low levels of PSA and PSMA expressions considering the principle of this assay, lymph nodes from females theoretically do not express these genes, indicating that the cutoff points used in this study were properly determined. Furthermore, in order to avoid underestimating the significance of micrometastases of prostate cancer cells, the expression levels of both PSA and PSMA mRNAs in each node, which were shown to be heterogenously expressed in prostate cancers (4), were measured, and nodes diagnosed as positively expressing PSA and/or PSMA mRNA were judged to be the presence of micrometastatic cancer foci. Collectively, these findings suggest that the present study was carried out under ideal conditions, which contributes to the reliability of the current outcomes.

We diagnosed the presence of occult micrometastasis in 225 lymph nodes from 43 patients using real-time RT-PCR assay, including 29 histologically involved nodes from 11 patients. This proportion of micrometastases to pelvic lymph nodes was significantly high compared with that reported in previous studies evaluated by RT-PCR (3, 4), suggesting that the real-time RT-PCR assay used in this study was more sensitive than conventional RT-PCR. In addition, the differences in the procedures between real-time RT-PCR and conventional RT-PCR contribute to the enhanced specificity; that is, it does not require post-PCR manipulation, and quantitation and calculation are all automated. In fact, immunohistochemical staining with PSA antibody detected micrometastatic cancer foci in approximately half of the pelvic nodes diagnosed as positive for micrometastasis despite the lack of histologic findings.

Characterization of clinicopathologic features according to nodal status showed that there were no significant differences in several conventional prognostic factors between patients with histologically detected nodal involvement and those with nodes positive for micrometastases despite the lack of histologically positive findings. Anatomic locations of micrometastatic nodes were also similar between these two patient groups. In addition, the proportion of patients positive for micrometastases was closely related to several poor prognostic indicators (data not shown). These findings strongly suggest that even with the lack of histologic confirmation, some of the

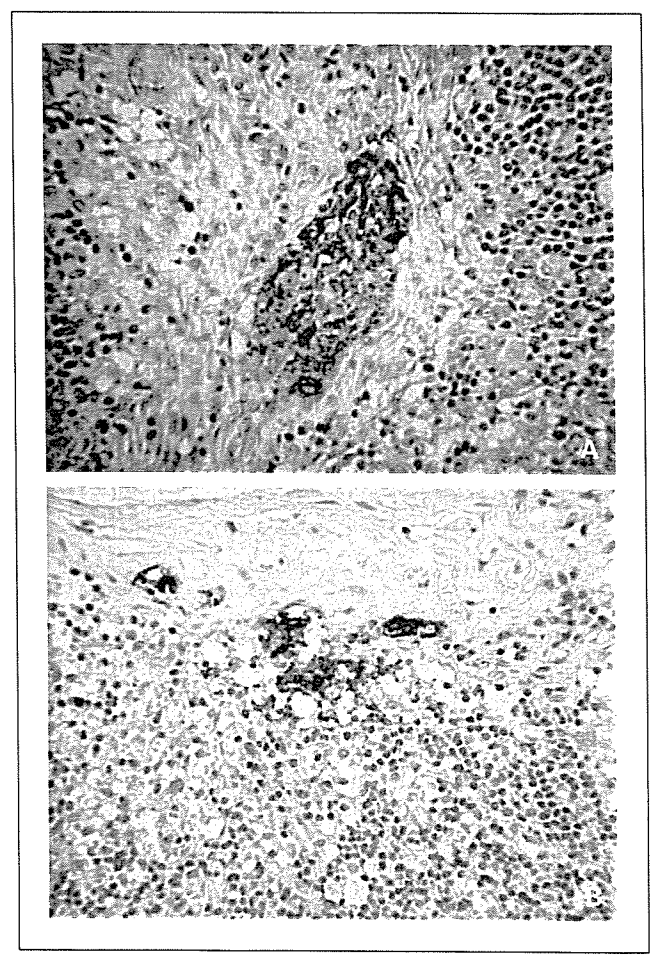

Fig. 2. Representative results of immunohistochemically detected micrometastatic cancer foci using a monoclonal antibody against PSA in histologically uninvolved lymph node specimens (*A* and *B*).

micrometastatic diseases diagnosed by the current real-time RT-PCR assay have biological characteristics similar to those of histologically positive nodal diseases. This hypothesis was supported by the incidence of biochemical recurrence following radical prostatectomy. Although the follow-up period of this study was too short to draw conclusions concerning the prognosis, there were no significant differences in the incidence of biochemical recurrence between these two groups. In addition, biochemical recurrence in four patients with pathologically organ-confined disease (diagnosed as positive for micrometastases) also supports this hypothesis. Furthermore, the presence of micrometastasis was independently associated with whether or not biochemical recurrence occurred—as well as the time to biochemical recurrence. Although longer follow-up periods are absolutely necessary to draw a definitive conclusion, the present findings suggest that some micrometastases in pelvic lymph nodes may, at least in part, contribute to the development of biochemical recurrence following radical prostatectomy.

To further address the significance of micrometastases in prostate cancer, several problems should be elucidated. For example, it would be of interest to investigate whether histologically undetectable or dormant micrometastatic disease in the lymphatic system will always progress to clinically significant recurrence after variable disease-free recurrence. If not, it will be necessary to develop a diagnostic system differentiating significant micrometastatic diseases from insignificant disease. Recent studies have reported the possible effect of lymphadenectomy on the survival of patients with pathologically confirmed positive nodes who underwent radical prostatectomy (18, 19). If there is a survival benefit in pelvic lymph node dissection for such patients, it would be interesting to evaluate whether removing micrometastatic nodes affects the prognosis. Recently, several investigators showed the usefulness of novel approaches for detecting occult prostate cancer metastases in lymph nodes (20, 21). For example, Shariat et al. reported that a splice variant-specific RT-PCR targeting the human glandular kallikrein gene can detect biologically and clinically significant micrometastases of prostate cancer in histopathologically normal lymph nodes (21). The assessment of these issues may facilitate the determination of a more appropriate procedure for lymphadenectomy considering the findings on molecular staging.

In conclusion, the results of this study showed the usefulness of quantitative real-time RT-PCR targeting the expression of PSA and PSMA genes for identifying micrometastatic tumor foci in pelvic lymph nodes from clinically localized prostate cancer at radical prostatectomy. Although longer follow-up periods are absolutely necessary to draw a definitive conclusion, the present findings suggest that some micrometastases in pelvic lymph nodes may, at least in part, contribute to the development of biochemical recurrence after radical prostatectomy.

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