a variety of apoptogenic proteins, most notably cytochrome *c*, SMAC/DIABLO, and HtrA2/Omi (9, 10). Cytosolic cytochrome *c* forms an apoptosome complex (11) with procaspase-9 and APAF1, which in turn releases active caspase-9. Like the extrinsic pathway, the intrinsic pathway converges on activation of caspase-3 (12). Tight regulation of caspase activation is required to prevent unchecked cell death. To this end, members of the inhibitors of apoptosis protein (IAP) family provide an intrinsic layer of antiapoptotic regulation. IAPs are an evolutionarily conserved protein family that functions to block cell death by binding to and inhibiting caspases (13, 14).

Eight human IAPs have been reported, namely, X-linked IAP (XIAP), cIAP1, cIAP2, survivin, NAIP, Apollon, Livin, and ILP-2 (15). The X-linked inhibitor of apoptosis, XIAP, is the best characterized of the IAP family members in terms of its potent caspase inhibitory mechanisms and is considered the prototype

Table 1. Relationship of XIAP protein expression with clinicopathologic parameters in prostate adenocarcinomas

	All patients	Mean XIAP expression (SE)	*P* (XIAP: continuous variable)*	Low XIAP intensity ≤1.8 (% of total)	High XIAP intensity >1.8 (% of total)	*P* (XIAP: dichotomized variable)†
Total cases (*N* = 192)		1.28 (0.041)		158 (82)	34 (18)	
Age at surgery						0.26 (NS)*
Median (range)	65 (46-76)			65 (46-76)	63.0 (50-75)	
Mean	63.8			64.0	63.0	
Gleason score			0.99 (NS)			0.31 (NS)
2-6	112 (58)	1.28 (0.055)		89 (56)	23 (68)	
7-10	80 (42)	1.27 (0.063)		69 (44)	11 (32)	
Pathology pT stage‡			0.63 (NS)			0.21 (NS)
PT2-pT3a	158 (82)	1.28 (0.046)		127 (80)	31 (91)	
PT3b	34 (18)	1.24 (0.092)		31 (20)	3 (9)	
Lymph node status (*n* = 190)			0.47 (NS)			>0.99 (NS)
Positive	11 (6)	1.12 (0.202)		9 (6)	2 (6)	
Negative	179 (94)	1.29 (0.042)		147 (94)	32 (94)	
Surgical margins			0.36 (NS)			0.55 (NS)
Positive	62 (32)	1.22 (0.076)		53 (34)	9 (26)	
Negative	130 (68)	1.30 (0.049)		105 (66)	25 (74)	
Capsular involvement			0.016§			0.11 (NS)
No invasion	40 (21)	1.10 (0.094)		34 (21)	6 (18)	
Invasion	113 (59)	1.38 (0.052)		88 (56)	25 (73)	
Extension	39 (20)	1.16 (0.090)		36 (23)	3 (9)	
Organ confined‖			0.15 (NS)			0.15 (NS)
Yes	100 (52)	1.33 (0.058)		78 (49)	22 (65)	
No	92 (48)	1.22 (0.058)		80 (51)	12 (35)	
High risk¶ (*n* = 190)			0.62 (NS)			0.28 (NS)
Yes	38 (20)	1.24 (0.090)		34 (22)	4 (12)	
No	152 (80)	1.29 (0.046)		122 (78)	30 (88)	
PreOpPSA, ng/mL (*n* = 172)						0.80 (NS)*
Median (range)	9.2 (0.6-96.5)			9.8 (0.6-76.0)	8.9 (3.2-96.5)	
Mean	14.0			14.0	14.0	
<10	87 (51)	1.31 (0.063)	0.74 (NS)			0.48 (NS)
≥10	85 (49)	1.31 (0.061)				
Recurrence**			0.082 (NS)††			0.0010††
Yes	69 (36)	1.18 (0.059)		67 (42)	2 (6)	
No	123 (64)	1.33 (0.055)		91 (58)	32 (94)	
Overall follow-up‡‡ (mo)						
Median (range)	78.5 (0.1-182.0)			74.0 (0.1-182.0)	88.5 (6.0-152.0)	
Mean	74.5			72.4	84.2	0.085 (NS)*
Total follow-up§§ (mo)						
Median (range)	48.5 (0.1-163.0)			41.0 (0.1-163.0)	87.0 (6.0-152.0)	
Mean	52.3			46.1	81.3	<0.0001*

**P* value was determined by the Mann-Whitney *U* test unless otherwise specified.
†*P* value was determined by the Pearson χ^2 test with Yates continuity correction unless otherwise specified.
‡pT3b indicates seminal vesicle invasion. There are no pT4 cases.
§*P* value was determined by the Kruskal-Wallis test. With capsular involvement as a continuous variable, *P* = 0.45 using the Spearman correlation corrected for ties.
‖No capsular extension and/or seminal vesicle and/or lymph node involvement. Margins are negative.
¶High-risk seminal vesicle and/or nodal positivity.
**Recurrence PSA elevation raising >0.2 ng/mL status post-radical prostatectomy.
††XIAP mean intensity association with recurrence by logistic regression of continuous data; (*P* = 0.082; 0.63; 95% confidence interval, 0.37-1.06), and of dichotomized data (*P* = 0.0010; 11.78; 95% confidence interval, 2.73-50.88). XIAP expression was the independent variable.
‡‡Overall follow-up time from primary surgery to last PSA follow-up.
§§Total follow-up time to recurrence to last follow-up in nonrecurrence.

of the IAP protein family (14, 16, 17). Abundant XIAP protein expression has been reported in a number of human cancers, including leukemia (18, 19), lymphoma (20), and tumors derived from prostate (4, 7, 21, 22), colon (23), lung (24, 25), cervical (26), bladder (4), hepatocellular (27), and vascular cells (28).

Here, we report that XIAP is elevated in prostate cancer and prostatic intraepithelial neoplasia (PIN) and is an independent predictor of cancer recurrence. Significantly, our results validate and greatly expand upon results by Krajewska et al. (4), showing a similar pattern. This finding provides further evidence that XIAP expression produces a counterintuitive direct association between expression and favorable clinical outcome implicating an as-yet undetermined set of coregulated mechanisms in this disease model. Nonetheless, the strong associations of XIAP expression to prostate cancer recurrence identifies it as a key molecule for targeted therapeutic investigation.

Materials and Methods

Patients. The study cohort consisted of 226 randomly selected hormone-naive patients who underwent radical retropubic prostatectomy between 1984 and 1995 as previously described (29–31). All prostate tumors were staged according to the 1997 American Joint Committee on Cancer tumor-node-metastasis staging system (32) and histologically graded using the Gleason scoring system (33). All cases were of the histologic type "adenocarcinoma, conventional, not otherwise specified" (34). Of the 226, 192 were informative for both recurrence outcomes and marker expression data. Table 1 shows the clinicopathologic data for this cohort.

Prostate tissue microarray construction. Formalin-fixed, paraffin-embedded archival tumor specimens were obtained from the University of California at Los Angeles Department of Pathology under Institutional Review Board approval. Case material was reviewed for tissue array construction by a study pathologist (D.S.). At least three core tissue biopsies (each 0.6 mm in diameter) were taken from morphologically representative regions of each prostate tumor and precisely arrayed as previously described (28–30). Tumor samples were accompanied by matching benign (morphologically normal or hypertrophic) and *in situ* neoplastic lesions (PIN), when available. Case material was arrayed into three tissue microarray (TMA) blocks. For staining, sections (5 μm) were transferred to glass slides using an adhesive slide system (PSA-CS 4, Instrumedics Inc.) to support cohesion of the array elements.

Immunohistochemistry. Immunohistochemical staining was done using an affinity-purified polyclonal rabbit anti-human/mouse XIAP antibody (R&D Systems, Inc.; Immunogen: aa 244-263 of human XIAP). A standard two-step indirect avidin-biotin complex (ABC) method was used (Vector Laboratories) as previously described (29, 30). PC-3 cells were used as a positive staining control for XIAP and were prepared as previously described (29). As a negative assay control, pooled nonimmune rabbit immunoglobulin G was applied at the same concentration as the anti-XIAP antibody.

Scoring of immunohistochemistry. Semiquantitative assessment of antibody on the TMAs was done by a study pathologist (H.Y.) blinded to the clinopathologic variables. The TMA was spot checked by a second pathologist (D.B.S.) for consistency of scoring. The target tissue for scoring was the glandular prostatic epithelium; scoring of benign tissues did not include basal cells. Tissue spot histology and grading were confirmed on the counterstained study slides. XIAP cytoplasmic expression was scored using two measures, intensity on a 0 to 3 scale (0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive) and percentage of positively stained target cells (range, 0-100% positive) staining at each intensity. To better represent overall protein levels, we combined the frequency and the intensity measures into an integrated intensity using the following formula: (% staining at intensity 3) × 3] + [(% staining at intensity 2) × 2] + [(% staining at intensity 1) × 1/100. To represent expression within cases, the mean pooled integrated intensity of the invasive tumor spots was used.

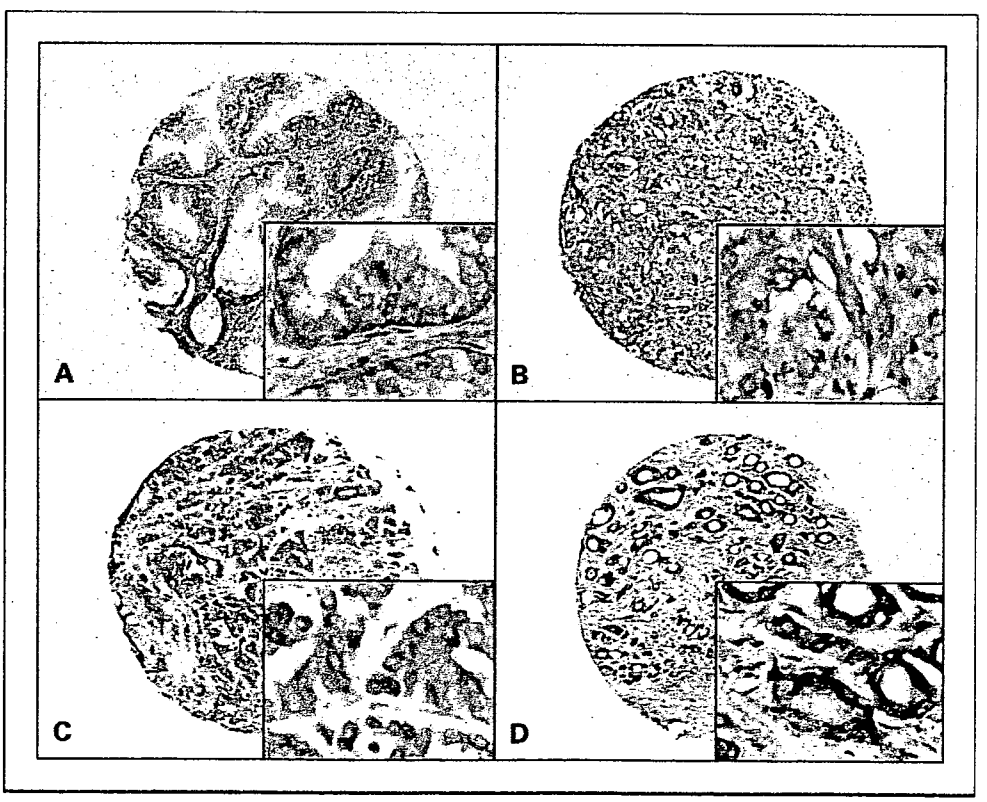

Fig. 1. XIAP protein expression in morphologically normal prostate and prostate cancer on tissue microarrays. Immunohistochemical staining for XIAP protein is seen on representative prostate tissue samples. *A*, normal tissue showing weak cytoplasmic epithelial staining of glandular cells. Staining in basal cells is frequently higher than that seen in glandular cells; scoring is from glandular cells. Invasive prostate cancers are shown demonstrating weak (*B*), moderate (*C*), and strong (*D*) cytoplasmic staining. Magnification, 100×, with 400× inserts.

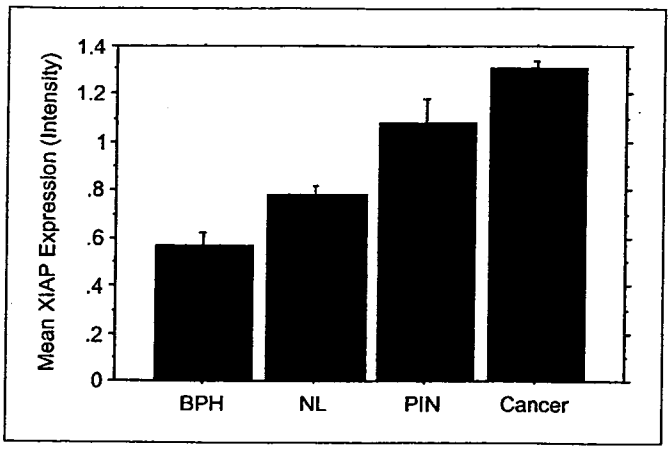

Fig. 2. XIAP protein expression distribution on the prostate tissue microarray stratified by histologic category. The intensity of XIAP protein expression in cells staining by immunohistochemistry as seen in 1,107 informative tissue microarray spots containing benign prostatic hyperplasia (*BPH*; $n = 122$), morphologically normal prostate (*NL*; $n = 252$), prostatic intraepithelial neoplasia (*PIN*; $n = 48$), and invasive prostate cancer (*Cancer*; $n = 685$) are shown as mean bar graphs. The mean XIAP expression was significantly higher in cancer (intensity = 1.32) compared with PIN (intensity = 1.08; $P = 0.019$), normal (intensity = 0.78; $P < 0.0001$), and BPH (intensity = 0.57; $P < 0.0001$). XIAP expression in PIN was significantly higher than normal ($P = 0.010$) and BPH ($P < 0.0001$), and expression in normal epithelium was significantly higher than that seen in BPH ($P = 0.0006$). The Mann-Whitney *U* test was used for two-group comparisons. Bars, 1 SE.

Statistical analysis. The Kruskal-Wallis and Mann-Whitney *U* tests were used to determine the significance of XIAP protein expression differences between categorical clinicopathologic prognostic variables. Associations of XIAP expression with continuous covariates were tested with the Spearman correlation. We used the Pearson χ^2 test to examine the association of dichotomized XIAP expression groups versus categorical variables. Recurrence was defined as a rising total PSA >0.2 ng/mL status post-prostatectomy, and time to recurrence was calculated from the date of the primary surgery. Patients without recurrence at last follow-up were censored. Kaplan-Meier plots were used to visualize recurrence-free time distributions, and the log-rank test was used to test for differences between them. We determined the optimal cut-point for dichotomized XIAP expression data using recursive partitioning, regression trees (rpart package), and plotting log-rank *P* values versus hazard ratios as previously described (35–37). An integrated intensity value of 1.8 gave a maximum hazard ratio and a minimal *P* value.

To assess which covariates associate with recurrence-free time, we fit both univariate and multivariate Cox proportional hazards regression models. The proportional hazards assumption was verified using Schoenfeld residuals (38). All *P* values were two-sided, and $P < 0.05$ was considered significant. All statistical analyses were done using R statistical software[10] and StatView version 5 (SAS Institute Inc.).

Results

XIAP protein expression in human prostate tissues. Using immunohistochemical techniques, we examined XIAP expression in human prostate tissue samples. Expression of XIAP in human prostate tissue was observed in the normal and malignant glandular epithelium, basal cells, and occasionally in stromal fibromuscular cells (Fig. 1). The human prostate cancer cell line, PC3, was used as a positive control for XIAP expression (data not shown). XIAP is typically expressed diffusely in the cytoplasm, but occasionally, discrete supranuclear staining in coarse clusters is additionally seen (Fig. 1).

[10] http://www.r-project.org/

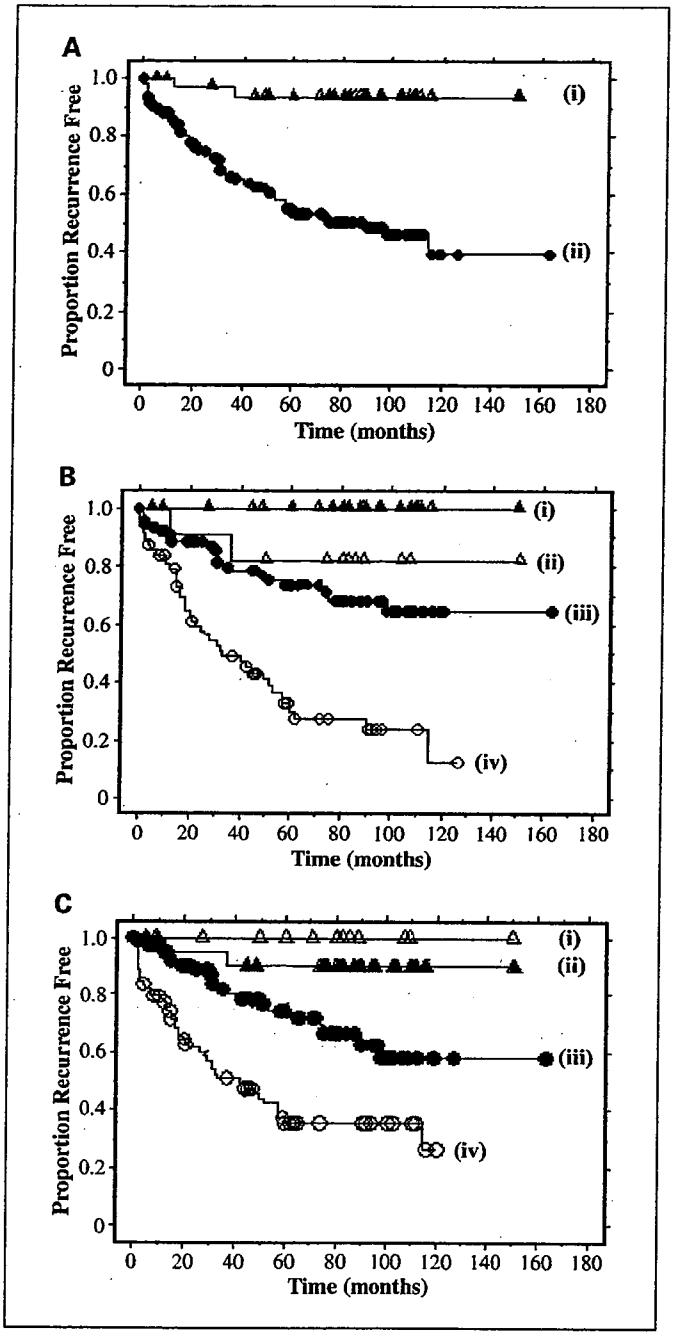

Fig. 3. Kaplan-Meier curves for time to prostate cancer recurrence. The high cytoplasmic XIAP expression phenotype is consistently associated with a lower risk of developing recurrent prostate cancer. For all figures, XIAP expression intensities of >1.8 and ≤1.8 are considered high and low XIAP, respectively. *A*, Kaplan-Meier curves for time to tumor recurrence stratified by cytoplamsic XIAP protein expression status ($n = 192$ patients) are seen in all patients. (*i*) High XIAP expression ($n = 34$); (*ii*) low XIAP expression ($n = 158$). Log-rank $P < 0.0001$. *B*, Kaplan-Meier curves in patients stratified by tumor grade. Gleason scores of 7 to 10 and 2 to 6 are considered high and low grade, respectively. (*i*) High XIAP, low grade ($n = 23$); (*ii*) high XIAP, high grade ($n = 11$); (*iii*) low XIAP, low grade ($n = 89$); (*iv*) low XIAP, high grade ($n = 69$). Log-rank $P < 0.0032$ for (*ii*) versus (*iv*). Log-rank $P < 0.0001$ for (*iii*) versus (*iv*) and for (*i*) versus (*iv*). There is no statistically significant difference between (*ii*) and (*iii*). *C*, Kaplan-Meier curves in patients stratified by their disease being organ confined with negative surgical margins ("confined") versus "not confined" (capsular extension and/or seminal vesicle involvement and/or lymph node involvement). (*i*) High XIAP, not confined ($n = 12$); (*ii*) high XIAP, confined ($n = 22$); (*iii*) low XIAP, confined ($n = 78$); (*iv*) low XIAP, not confined ($n = 80$). Log-rank $P < 0.041$ for (*ii*) versus (*iii*). Log-rank $P < 0.0001$ for (*ii*) versus (*iv*), (*i*) versus (*iv*), and (*iii*) versus (*iv*). For all figures, censored times are marked by either circles or triangles.

Table 2. Cox proportional hazards analysis for time to PSA recurrence

Variable	Univariate* (all patient†, n = 192)	Multivariate* (all patients‡, n = 172)		Univariate* (low Gleason§, n = 112)
		Continuous	Dichotomized	
Gleason score >7	<0.0001	0.0011	0.0014	NA
	3.70 (2.23-6.11)	2.81 (1.51-5.24)	2.80 (1.49-5.26)	
Seminal vesicle invasion (stage = pT3b)	<0.0001	0.0035	0.0032	0.0065
	4.10 (2.47-6.81)	2.46 (1.35-4.51)	2.46 (1.35-4.47)	5.52 (1.61-18.89)
Capsular invasion	0.0038	0.019	0.036	0.014
	1.73 (1.19-2.52)	1.67 (1.09-2.57)	1.55 (1.03-2.35)	2.21 (1.17-4.16)
Preoperative PSA	0.015	0.60	0.70	0.024
	1.02 (1.00-1.03)††	1.00 (0.99-1.02)	1.00 (0.99-1.02)	1.04 (1.01-1.07)‡‡
XIAP intensity (continuous)‖‖	0.033	0.077	NA	0.028
	1.54 (1.04-2.29)	1.49 (0.96-2.33)		2.20 (1.09-4.44)
XIAP intensity ≤1.8 (dichotomized)¶¶	0.0010	NA	0.0025	**
	10.69 (2.61-43.73)		8.92 (2.16-38.86)	

*P value; hazard ratio; (95% confidence interval) provided.
†64% of cases are censored.
‡67% of cases are censored.
§Gleason score 2 to 6; 79% of cases are censored.
‖Gleason score 2 to 6; 83% of cases are censored.
¶Gleason score 7 to 9 (no Gleason score 10 cases are present); 43% of cases are censored.
**High XIAP group has no events (all patients are censored).
††n = 172.
‡‡n = 103.
§§n = 69.
‖‖Pooled mean XIAP intensity. Used formula (3 − continuous XIAP intensity) to reverse hazard ratio to compare directly to other covariates. A high XIAP carries a reduced risk of recurrence.
¶¶Pooled mean XIAP intensity dichotomized: ≤1.8 (n = 158); >1.8 (n = 34).

Basal cells in normal glands are frequently stained more strongly than the glandular cells. Our scoring of benign epithelium was limited to these glandular cells.

We examined the XIAP expression distribution stratified by histologic category (Fig. 2). Notably, XIAP is elevated in prostate cancer versus matching benign tissues; this increase can be seen starting in PIN lesion. Regions of benign prostatic hyperplasia (BPH) showed the lowest expression. The intensity of XIAP staining are shown in Fig. 2. The mean XIAP expression was significantly higher in cancer (intensity = 1.32) compared with PIN (intensity = 1.08; $P = 0.019$), normal (intensity = 0.78; $P < 0.0001$), and BPH (intensity = 0.57; $P < 0.0001$). In addition, XIAP expression in PIN was significantly higher than normal ($P = 0.010$) and BPH ($P < 0.0001$), and expression in normal epithelium was significantly higher than that seen in BPH ($P = 0.0006$). We found no significant difference in XIAP expression when broken down by tumor grade or Gleason score (data not shown).

XIAP expression and cancer recurrence. We next examined the potential association XIAP protein expression with tumor recurrence following radical prostatectomy. Recurrence data were available for 192 XIAP-informative cases. Case-level expression was derived by pooling the mean integrated intensities of the spots as previously reported (38). Supervised survival tree analysis was applied to pooled data, and a dichotomized population was defined with an optimal cut-point of 1.8 mean integrated intensity representing individuals with higher versus lower XIAP expression. Specifically, an expression intensity of >1.8 was considered "Higher XIAP expression", and ≤1.8 was considered "Lower XIAP expression".

We examined the association of XIAP as either a continuous or dichotomized variable with established prognostic factors and found that expression of XIAP was associated with disease recurrence (Table 1). Figure 3A shows a Kaplan-Meier estimate of cancer recurrence-free time stratified by XIAP expression. Significantly, the median recurrence-free time was 75 months for cases with low XIAP, compared with >152 months for cases with high XIAP ($P < 0.0001$).

Cox proportional hazards analyses were done for established prognostic factors and time to PSA recurrence (Table 2). Of particular note is the strength of XIAP predictive power as a dichotomized variable, which was higher in all cases than the conventional prognosticators. Higher XIAP expression predicted a reduced risk of tumor recurrence both as a continuous ($P = 0.033$) and a dichotomized ($P = 0.0010$) variable in univariate analysis. The dichotomized XIAP remains highly significant in multivariate analysis in this category ($P = 0.0025$), as well as after substratifying by Gleason score ($P = 0.010$ for high-grade cases). Significantly, in patients with primary low-grade cancer, no individuals who had high levels of XIAP had tumor recurrence ($n = 23$). In contrast, 26% of individuals with low-grade cancer who had low levels of XIAP had tumor recurrence ($n = 89$). Figure 3B shows XIAP expression further substratified by Gleason score, and Fig. 3C shows XIAP expression further substratified by whether or not the tumor is organ confined. Significantly, higher XIAP portends a good outcome regardless of the grade or organ confinement status; patients with higher grade or non-organ-confined tumors with higher XIAP expression do better as a group than any patient whose tumors express

Table 2. Cox proportional hazards analysis for time to PSA recurrence (Cont'd)

Multivariate* (low Gleason‖, n = 103)		Univariate* (high Gleason¶, n = 80)	Multivariate* (high Gleason¶, n = 69)	
Continuous	Dichotomized		Continuous	Dichotomized
NA	NA	NA	NA	NA
0.037	**	0.0086	0.012	0.0089
4.07 (1.09-15.20)		2.21 (1.22-3.98)	2.36 (1.21-4.60)	2.45 (1.25-4.80)
0.0049	**	0.42	0.52	0.69
3.08 (1.41-6.73)		1.23 (0.75-2.04)	1.20 (0.69-2.09)	1.11 (0.66-1.86)
0.011	**	0.95	0.84	0.67
1.04 (1.01-1.08)		1.00 (0.98-1.02)§§	1.00 (0.98-1.02)	1.00 (0.98-1.02)
0.17	NA	0.25	0.19	NA
1.85 (0.77-4.43)		1.33 (0.82-2.17)	1.42 (0.84-2.41)	
NA	**	0.011	NA	0.010
		6.37 (1.54-26.43)		6.61 (1.57-27.89)

low XIAP, even those of low grade or that are organ confined (Fig. 3B and C).

Of note, the high predictive value of XIAP in the specific substrata described above generate subgroups in which 100% of the population was without tumor recurrence (Fig. 3B and C). Because of this, no Cox P values can be calculated in these statistical models. However, Table 3 shows how effectively XIAP stratification can isolate low-recurrence groups in all patient substrata examined. For example, in patients whose tumors were not organ confined (n = 92), 50% experienced disease recurrence. However, within this group, none of the 12 patients with high XIAP expression tumors experienced recurrence.

Discussion

The IAP family member XIAP is the strongest direct inhibitor of caspases and is therefore a significant downstream anti-apoptotic protein. Aberrant expression of XIAP has been implicated in the pathology of a number of human cancers; however, few large-scale *ex vivo* studies have been done, and fewer provide translational associations of XIAP expression levels to clinical outcomes.

In support of the role of XIAP as an apoptosis inhibitor, we find that the level of XIAP expression is higher overall in prostate cancer as compared with matched benign tissues, with an intermediate expression observed in PIN. These findings are in agreement with other studies, suggesting that XIAP helps to promote tumor cell survival. Pathologically elevated XIAP levels have been found in a number of hematologic (19, 20, 40–42), vascular (28), and epithelial (4, 23–25, 27) malignancies, as well as in most cell lines of the NCI-60 tumor screening panel (40, 43). Only rare exceptions to this pattern have been reported (26).

We further examined the potential association of XIAP expression with clinicopathologic parameters. Paradoxically, when dichotomized optimally, lower levels of XIAP expression were a strong predictor of recurrence, whereas higher expression strongly predicted a substantially reduced risk of recurrence. In fact, XIAP generated a larger hazard ratio (i.e., stronger predictive power) than those seen from conventional prognostic indicators, including Gleason score, tumor stage capsular invasion, and preoperative PSA. As demonstration of its predictive power, patients with high-grade metastatic tumors and high XIAP had a lower risk of recurrence than patients with low-grade nonmetastatic tumors and with low XIAP. Strikingly, no patients with low-grade tumors plus high XIAP levels had tumor recurrence. In contrast, more than 25% of patients with low XIAP expression experienced recurrences. Despite having a longer overall PSA follow-up, 94% of all patients with high XIAP expression were recurrence-free at the end of follow-up, versus 58% of patients with low XIAP tumors. These findings,

Table 3. Prostate cancer recurrence status in patient groups and substratified by XIAP protein expression category

Patient group	Total count (n)	Total % censored*	Low XIAP† % censored (count, n)	High XIAP† % censored (count, n)
All patients	192	64	58 (158)	94 (34)
Low grade‡	112	79	74 (89)	100 (23)
High grade	80	43	36 (69)	82 (11)
Organ confined§	100	77	73 (78)	91 (22)
Not confined	92	50	42 (80)	100 (12)

*Proportion of patients who reach the end of PSA follow-up without evidence of recurrence. Recurrence = PSA elevation raising >0.2 ng/mL status post-radical prostatectomy.
†Pooled mean XIAP intensity dichotomized: low ≤1.8; high >1.8 on a 0 to 3 scale.
‡Low grade = Gleason score of 2 to 6; high grade = Gleason score of 7 to 9 (there are no cases of Gleason 10 in this cohort).
§Organ confined = no capsular extension and/or seminal vesicle and/or lymph node involvement. Margins are negative.

coupled to the lack of direct association with any of the clinicopathologic variables tested, shows the independence XIAP and its widespread applicability as a prognostic indicator.

Our current study confirms the work of Krajewska et al., who also found that high levels of XIAP were associated with a reduced risk of recurrence in prostate cancer patients (4). The importance of independent validation for tumor biomarkers cannot be overemphasized because such verification is an absolute requirement for differentiating biomarkers, which have the potential to be meaningful clinical predictors from those that demonstrate merely idiosyncratic expression (44–46). In addition, such validation studies are also critical to minimize overfitting of statistical data. Therefore, that the predictive power of XIAP was observed in two separate and independent patient populations is highly significant.

The results shown here not only validate the findings of Krajewska et al., but it also extends their work (4). To our knowledge, our study is the largest study to date examining the association of XIAP protein to clinical outcomes. Moreover, the patient cohort for clinical outcomes in the aforementioned study (4) consisted of needle core biopsies from 64 $T_2N_0M_0$ radiation-treated patients. Here, we provide an expanded and unrelated patient population on tissue microarrays to include 192 informative patients with a spectrum of disease stages. The only other major difference between the two studies is that our results suggest that XIAP is an independent predictor of outcome, whereas Krajewska et al. found a significant inverse correlation of XIAP with preoperative PSA level; they offered this as a potential link to the positive outcome seen in high XIAP-expressing patients.

XIAP expression in other malignancies. The association of high XIAP expression with a positive clinical outcome is counterintuitive to expectations that IAPs promote tumor cell survival. Nevertheless, some recent studies of lung cancer have shown that increased levels of XIAP are associated with an improved prognosis (4, 47). For example, Ferriera et al. (47) found that higher levels of XIAP correlated with longer survival in early-stage non–small cell lung cancer (NSCLC) patients. Surprisingly, the same group found that XIAP was not associated with survival in advanced NSCLC (24).

Conversely, several studies have shown a negative association of XIAP levels to outcomes (cancer recurrence/remission and/or death) in other types malignancies. For example, XIAP expression was found in 95% of clear cell renal cell carcinomas (48). A significant increase was observed from well to poorly differentiated tumors. Tamm et al. and Carter et al. (18, 40–42) found that in patients with acute myelogenous leukemia, higher levels of XIAP correlated with a slightly shorter remission durations and a decreased survival time. Several other studies failed to find associations between XIAP levels and survival, including those focusing on colon (23), cervical (26), and bladder cancers (4); the latter two studies also noted a lack of association of XIAP with tumor grade and stage.

Potential mechanism of action. The observation that XIAP is elevated in primary prostate tumor cells, yet also high levels ultimately predict a lower probability of tumor recurrence, is intriguing. There are a number of possible explanations for these observations. XIAP has been reported to mediate cell cycle arrest via down-regulation of cyclins A and D1 and induction of cyclin-dependent kinase inhibitors p21Cip1/Waf1 and p27Kip1 (28). Thus, although XIAP may provide a selective antiapoptotic survival advantage, it may simultaneously impair the proliferation of cancer cells. It is possible that these two properties function with some degree of independence.

XIAP is itself regulated by antagonists such as SMAC/DIABLO, which is released from the mitochondria upon apototic stimuli (12, 49–51). Recent studies have shown that the relative proportion of XIAP compared with SMAC/DIABLO is the factor that dictates life versus death decisions. Therefore, it is possible that the high levels of XIAP expression seen in our study are counteracted by higher levels of anti-IAPs. We are currently exploring this possibility.

Finally, as is the case with all studies involving immunohistochemistry on archival paraffin-embedded sections, the overall activity of XIAP cannot be assessed. Whether XIAP functions differently in a progressing tumor cell and/or interacts with alternate target molecules in an evolving malignant cell is an intriguing possibility that warrants further study.

Malignant prostate cancer remains a disease with few useful outcome measures and no current consistently effective therapies. Therefore, informative biomarkers are urgently needed to guide patient surveillance and clinical intervention. This study reports the overexpression of XIAP in primary human prostate cancers and provides strong evidence for its beneficial prognostic association.

Acknowledgments

We thank Stephanie Hana and Greg Kanter for technical assistance and Rebecca Schatz for constructive comments.

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Prognostic Significance of Thymidylate Synthase Expression in Patients with Prostate Cancer Undergoing Radical Prostatectomy

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OBJECTIVES Thymidylate synthase (TS), a key enzyme in DNA synthesis, is overexpressed in a variety of cancer cells. 5-Fluorouracil (5-FU), an anticancer agent used clinically against various cancers, including prostate cancer, inhibits DNA synthesis by binding TS. In this study, we investigated the expression of TS in prostate cancer and its prognostic significance. Its association with the expression of dihydropyrimidine dehydrogenase (DPD), a principal enzyme in the degradation of 5-FU and pyrimidine nucleotides, was also examined.

METHODS Fifty-two prostatic tissue specimens were obtained from patients who had undergone radical prostatectomy for prostate cancer without neoadjuvant hormonal therapy. We analyzed the cancerous tissue and normal prostatic tissue specimens for TS expression using immunohistochemistry.

RESULTS TS was expressed at greater levels in the prostate cancer specimens than in the normal prostatic tissue specimens. The patients with prostate cancer with negative TS expression had a longer postoperative recurrence-free rate than did those with positive expression during the 5 years of follow-up. TS expression was significantly decreased in patients who received neoadjuvant hormonal therapy. No relationship was found between the expression of TS and DPD. Patients with prostate cancer with either negative TS or DPD expression had a significantly longer postoperative disease-free rate than those with positive expression of both during the 5 years of follow-up.

CONCLUSIONS The results of the present study have shown for the first time that TS expression could be a prognostic marker for patients with prostate cancer undergoing radical prostatectomy. In addition, the combination of TS and DPD expression might also be helpful for the prediction of the prognosis of patients with prostate cancer. UROLOGY 69: 988–995, 2007. © 2007 Elsevier Inc.

The anticancer agent, 5-fluorouracil (5-FU), is used clinically against various cancers, including prostate cancer.[1,2] Single-agent infusion 5-FU has demonstrated some efficacy against hormone-refractory prostate cancer, and response rates up to 27% have been reported.[3] 5-FU itself is inactive and requires intracellular conversion to 5-fluoro-2′-deoxyuridine 5′-monophosphate. 5-Fluoro-2′-deoxyuridine 5′-monophosphate exerts its cytotoxic activity through the formation of a ternary complex with thymidylate synthase (TS) and 5,10-methylene-tetrahydrofolate, resulting in inhibition of TS and blockage of the DNA synthetic process.[4,5] TS is overexpressed in tumor cells, which show high proliferative activity.[6] Several studies examining the importance of TS expression have indicated that TS expression predicts for overall outcome and the response to 5-FU cytotoxic therapy in several major tumor types.[7–9] Furthermore, the immunohistochemical staining results for TS and dihydropyrimidine dehydrogenase (DPD) predict the response to 5-FU.[10,11]

Our previous studies on renal cell carcinoma and bladder cancer showed that TS activity was greater in the cancerous tissue specimens than in the normal tissue samples and that

This work was supported in part by the NPO Tsukusi Fellowship and Research Foundation.

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Submitted: May 4, 2006, accepted (with revisions): February 8, 2007

0090-4295/07/$32.00
doi:10.1016/j.urology.2007.02.015

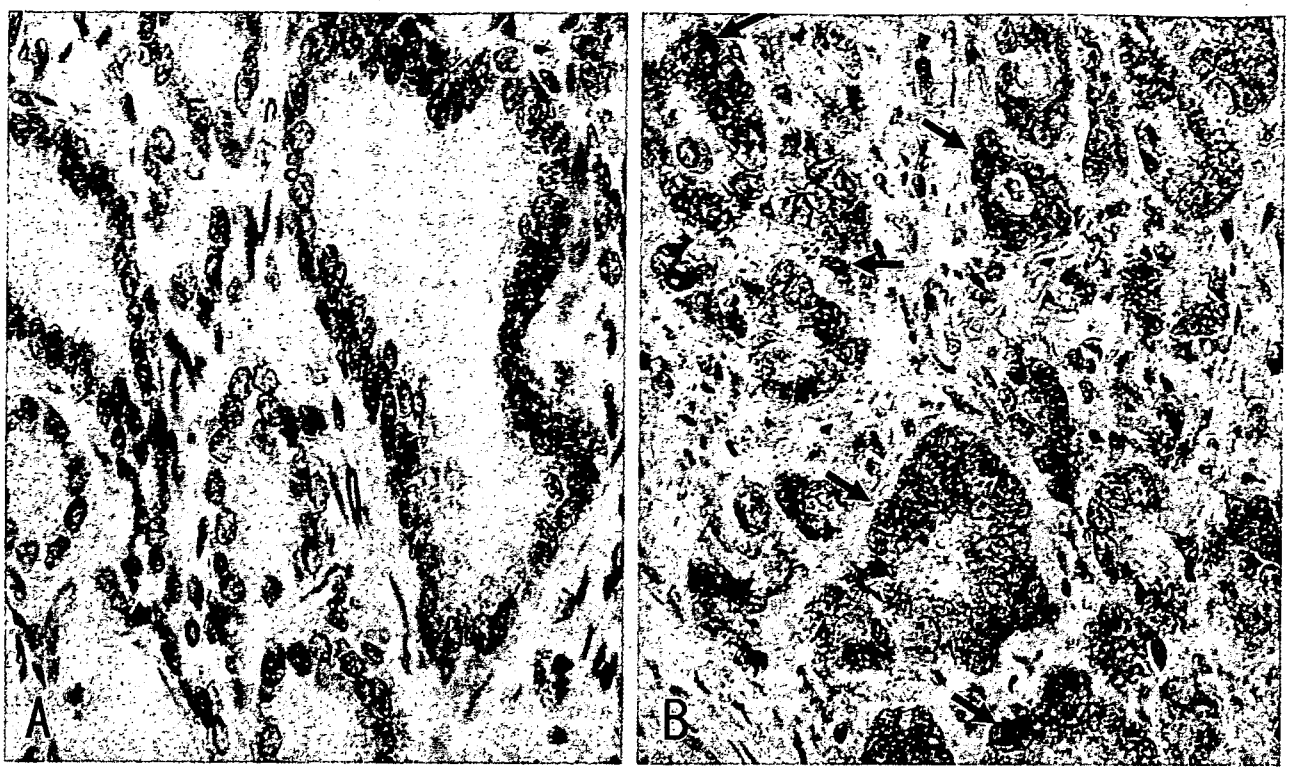


Figure 1. Immunohistochemical staining for TS in normal prostatic tissues and prostate cancer. Representative images of tissue samples with absent and strong TS expression were examined. TS expression confined to cytoplasm of cells as demonstrated by immunohistochemistry. Original magnification ×200. **(A)** Negative TS staining in normal prostatic tissue. **(B)** Positive TS staining (arrows) observed in cytoplasm of prostate cancer tissue.

the TS activity level correlated with stage progression and increase in bladder cancer grade.[12] In addition, TS activity is a significant prognostic marker in patients with bladder cancer or renal cell carcinoma.[12,13] Reported data on TS activity in prostate cancer are limited, and little is known about the significance of TS in the biology of prostate cancer. The aim of this study was to define whether TS expression is a prognostic marker for patients with prostate cancer.

MATERIAL AND METHODS

Patients

We obtained 52 prostate cancer specimens with adjacent normal prostatic tissues from 1997 to 2005. The patients had not undergone preoperative androgen-deprivation therapy or radiotherapy. The mean patient age was 65 years (range 53 to 75). The 2002 TNM system was used for pathologic staging.[14] The pathologic stage was T2 in 38 patients and T3 in 14. The Gleason grading system was used to determine the Gleason score.[15] The Gleason score of the 52 specimens was grade 4/5 in 1 patient, 4/4 in 1, 4/3 in 9, 3/5 in 2, 3/4 in 18, 3/3 in 8, 3/2 in 8, 2/3 in 3, 2/1 in 1, and 1/2 in 1 patient. In addition, prostate cancer tissue from 48 patients who had undergone neoadjuvant hormonal therapy was examined.

The local human investigations committee approved this study, and all patients provided informed consent.

Immunohistochemistry for TS and DPD

TS and DPD expression was examined by immunohistochemistry, as previously described.[5,16] The sections were incubated with monoclonal antibody TS106 (1:500, dilution, Taiho Pharmaceutical, Saitama, Japan) or incubated with polyclonal rabbit antibody against human DPD[16] (1:2000 dilution, Taiho Pharmaceutical) overnight at 4°C. The secondary antibody was visualized with diaminobenzedine.

Evaluation of TS and DPD Expression

The intensity of the immunoreactivity for TS and DPD was evaluated in normal prostatic tissue and prostate cancer tissue from the same slide in each case. At least 10 high-power fields at 400× magnification were chosen randomly, and more than 1000 carcinoma cells were counted for each section. A pathologist who was unaware of the clinicopathologic data and clinical outcomes of the patients examined cytoplasmic TS and DPD staining results. The intensity of TS and DPD was graded from 0 to 3, and the extent was graded as focal (less than 25% of tumor staining positive) or diffuse (more than 25% of tumor staining positive).[5] A score of 0 or 1 was regarded as negative expression, and a score of 2 or 3 as positive expression. Figure 1 shows representative examples; Fig. 1A shows a TS-negative normal prostate specimen and Fig. 1B TS-positive prostate cancer.

Statistical Analysis

For statistical analysis, the Student *t* test and chi-square test were used. Biochemical recurrence was defined as a postopera-

tive serum prostate-specific antigen (PSA) level of 0.1 ng/mL or more.[17] The postoperative recurrence-free rate was determined using the Kaplan-Meier method. The influence of each variable on the recurrence-free rate was analyzed by multivariate analysis of a Cox proportional hazard model. $P < 0.05$ was considered significant.

RESULTS

TS Expression in Prostate Cancer and Normal Prostatic Tissue

TS was expressed in the cytoplasm of both normal prostatic tissue and prostate cancer cells. TS expression was detected in 35 (67%) of 52 prostate cancer samples (Fig. 2A). In contrast, TS expression was detected in 5 (9%) of the 52 normal prostatic tissue specimens. In addition, the intensity of cells that reacted with TS antibodies was significantly greater in the prostate cancer specimens than in the normal prostate samples ($P < 0.0001$, data not shown).

TS Expression in Relation to Pathologic Features and Tumor Stage

The staining percentage of TS expression was greater in patients with Gleason score 7 or greater disease (26 of 31, 83%) than that for patients with Gleason score less than 7 disease (9 of 21, 42%; Fig. 2B). It was also greater in Stage T3 tumor (14 of 14, 100%) than in Stage T2 tumors (21 of 38, 55%; Fig. 2C).

Relationship Between TS Expression and Postoperative Recurrence-Free Rate in Patients with Prostate Cancer

Patients with prostate cancer undergoing radical prostatectomy alone were evaluated to determine the postoperative clinical course. From these results, patients with prostate cancer were divided into two groups—those with positive TS expression and those with negative TS expression. At 5 years of follow-up, patients with negative TS expression had a greater recurrence-free rate compared with those with positive TS expression ($P = 0.0183$; Fig. 3A). Using Cox regression analysis for the 52

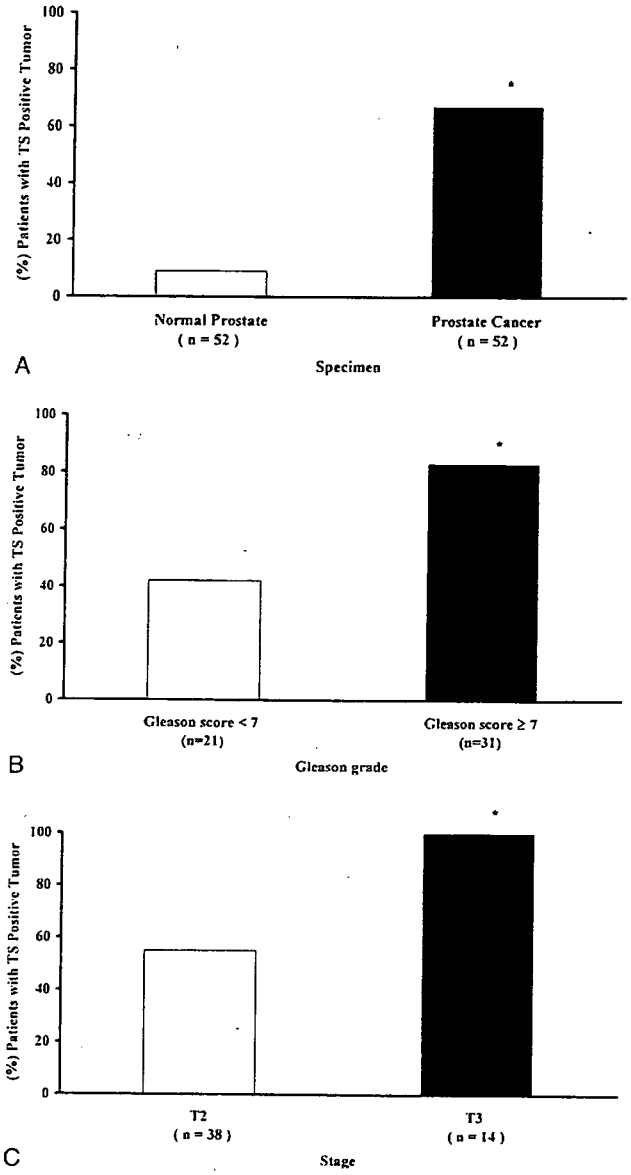
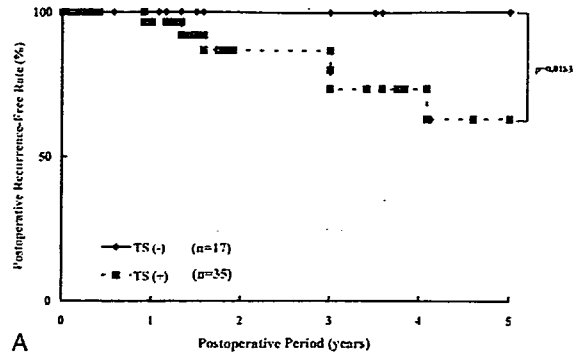
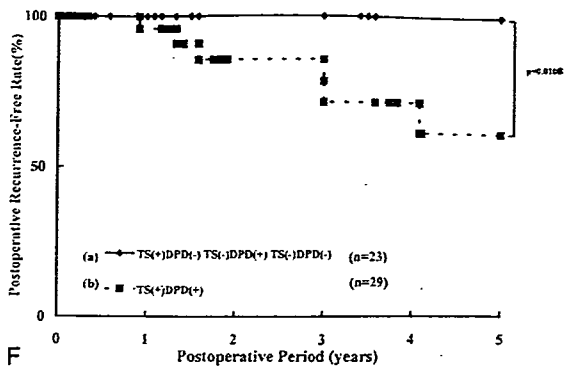


Figure 2. Expression of TS in normal prostatic tissue and prostate cancer. Percentage of TS expression detected by immunohistochemical assay as described in Material and Methods section. **(A)** $^*P < 0.0001$ versus normal prostatic tissue; **(B)** $^*P = 0.0017$ versus Gleason score less than 7 disease; and **(C)** $^*P = 0.0113$ versus Stage T2.

Figure 3. Relationship between TS expression and postoperative recurrence-free rate in patients with prostate cancer and relationship between TS and DPD expression and postoperative recurrence-free rate in patients with prostate cancer. Postoperative recurrence-free rate of 52 patients with prostate cancer undergoing radical prostatectomy alone was determined using Kaplan-Meier method. **(A)** Patients categorized by TS expression. Recurrence-free rate was significantly greater for patients with negative TS expression than for those with positive expression ($P = 0.0183$). **(B)** Patients categorized by TS expression of Gleason score less than 7 disease. Recurrence-free rate was significantly greater for patients with negative TS expression than for those with positive expression ($P = 0.0256$). **(C)** Patients categorized by TS expression of Gleason score 7 or worse. No significant difference observed between two groups of patients ($P = 0.2722$). **(D)** Patients categorized by TS expression of preoperative serum PSA level of 10 ng/mL or less. No significant difference observed between two groups of patients ($P = 0.2296$). **(E)** Patients categorized by TS expression of preoperative serum PSA level greater than 10 ng/mL. Recurrence-free rate significantly greater for patients with negative TS expression than for those with positive expression ($P = 0.0344$). **(F)** Patients (n = 52) categorized as having positive expression of both TS and DPD (b) and all others (a). Significant difference in recurrence rate between two groups ($P = 0.0108$). **(G)** Patients with Gleason score less than 7 disease. **(H)** Patients with Gleason score of 7 or more. **(I)** Patients with tumor with preoperative serum PSA level of 10 ng/mL or less. **(J)** Patients with tumor and preoperative serum PSA level greater than 10 ng/mL.

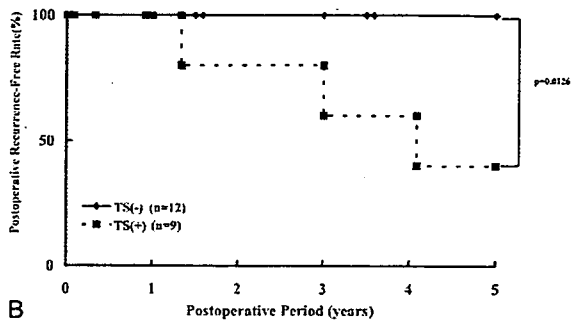


A



F

Gleason score < 7



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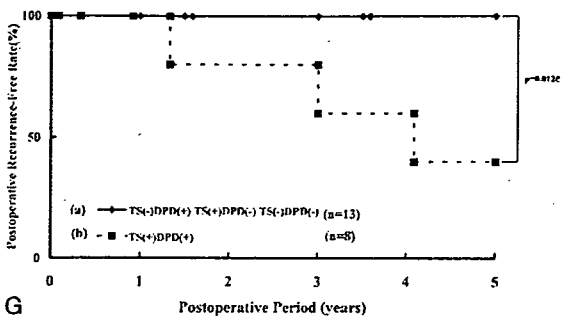
Gleason score < 7



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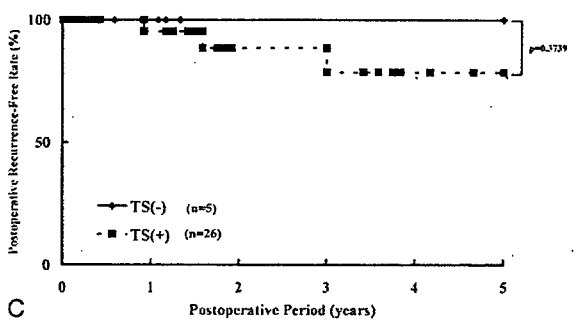

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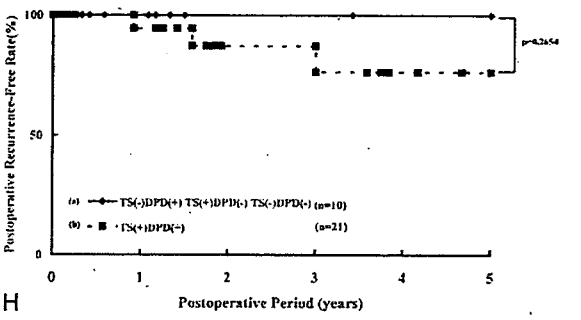
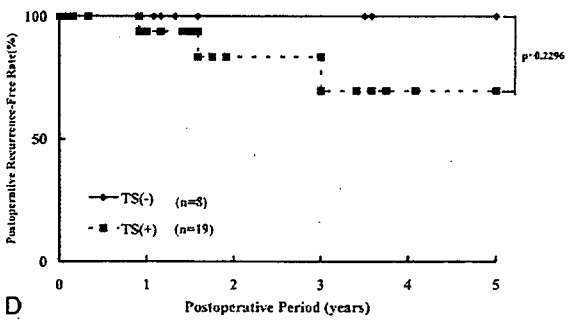


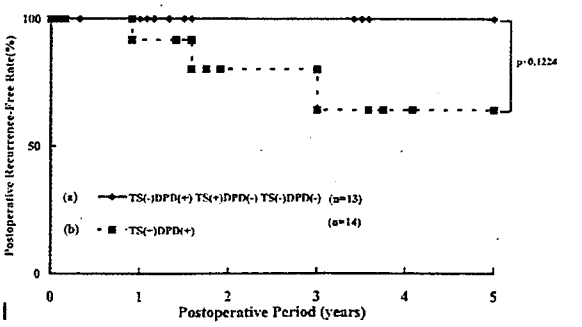
H

Pre-operative serum PSA ≦ 10



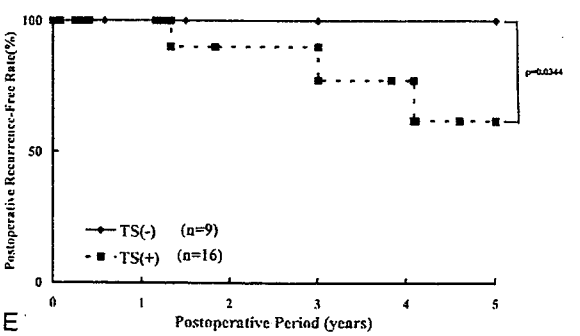
D

Pre-operative serum PSA ≦ 10



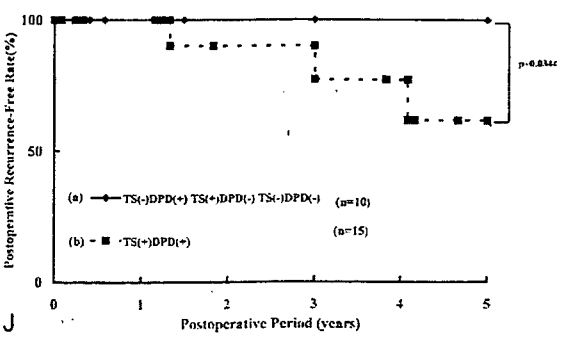
I

Pre-operative serum PSA > 10



E

Pre-operative serum PSA > 10



J

patients, TS expression seemed to be an independent prognostic indicator ($P = 0.021$ on multivariate analysis). The patients were also divided according to the Gleason grade and preoperative serum PSA level at baseline, and the recurrence-free rate of the groups with different TS expression status was analyzed. In Gleason score less than 7 cancer, the TS-negative group had a significantly greater 5-year recurrence-free rate than did the TS-positive group (Fig. 3B). In those with Gleason score 7 or greater cancer, no significant difference was found (Fig. 3C). In those with a PSA of 10 ng/mL or less at baseline, the 5-year recurrence-free rate of TS-negative patients tended to be greater than that of TS-positive patients. However, statistical significance was not reached (Fig. 3D). In those with a PSA level greater than 10 ng/mL, the TS-negative group had a significantly greater 5-year recurrence-free rate compared with the TS-positive group (Fig. 3E). These findings indicate that the TS expression level in prostate cancer could be a prognostic indicator, with negative TS expression a good prognostic sign.

Effect of Neoadjuvant Hormonal Therapy on TS Expression in Prostate Cancer

The expression of TS in patients with prostate cancer was greater after radical prostatectomy alone than that after radical prostatectomy plus neoadjuvant hormonal therapy (32 of 52, 61% versus 18 of 48, 37%; Fig. 4A). For patients with Stage T2 prostate cancer, positive TS expression in those who underwent neoadjuvant hormonal therapy (9 of 38, 23%) was less than that in patients who underwent radical prostatectomy alone (21 of 38, 55%; Fig. 4B). However, no significant difference was found in those with Stage T3 prostate cancer (14 of 14, 100% versus 10 of 10, 100%; Fig. 4C). With Gleason score less than 7 prostate cancer, positive TS expression in patients with radical prostatectomy alone (9 of 21, 42%) was significantly greater than that in patients who had undergone neoadjuvant hormonal therapy (1 of 13, 7%; Fig. 4D). A significant difference was also observed in patients with Gleason score 7 or greater prostate cancer between those who underwent radical prostatectomy alone (26 of 31, 83%) and those who also received neoadjuvant hormonal therapy (18 of 35, 51%; Fig. 4E).

These findings suggest that neoadjuvant hormonal therapy might downregulate TS expression in prostate cancer, especially Stage T2 prostate cancer and Gleason score less than 7 disease.

Prognostic Significance of Combined TS and DPD Evaluation

DPD is the initial and rate-limiting enzyme in the three-step pathway of pyrimidine nucleotide catabolism.[18] In contrast, TS is an important enzyme in pyrimidine nucleotides synthesis. Kornmann *et al.*[19] reported that the combination of TS and DPD expression correlated highly with survival. We examined the association between TS and DPD expression in patients with prostate cancer. No significant association was observed between the levels of TS and DPD expression using the chi-square test (data not shown).

We then examined the prognostic significance of a combination of TS and DPD expression using Kaplan-Meier analysis. On the basis of TS and DPD expression, the 52 patients were stratified categorized as those having positive expression of both TS and DPD and all others. We found a significant difference between these two groups ($P = 0.0108$; Fig. 3F). Patients with positive expression of both TS and DPD had a greater rate of postoperative recurrence. The patients also were divided using the Gleason grade and preoperative serum PSA level at baseline, and the recurrence-free rate of the groups with different TS and DPD expression were analyzed. In those with Gleason score less than 7 cancer, the TS-negative group had a significantly greater 5-year recurrence-free rate than did the TS-positive group (Fig. 3G). In patients with Gleason score 7 or greater cancer, no significant difference was found (Fig. 3H). In patients with a PSA level of 10 ng/mL or less, the 5-year recurrence-free rate of the TS-negative group tended to be greater than that of the TS-positive group. However, statistical significance was not reached (Fig. 3I). In those with a PSA level greater than 10 ng/mL, the TS-negative group had a significantly greater 5-year recurrence-free rate than that of the TS-positive group (Fig. 3J).

COMMENT

The role of TS expression in prostate cancer has not been previously studied. Multivariate analysis revealed that the TS expression profile was an independent prognostic indicator of prostate cancer. These results suggest that TS expression in patients with prostate cancer might provide additional prognostic information beyond the orthodox clinical and pathologic prognostic markers. Ichikawa *et al.*[20] reported that patients with colorectal tumor with both low DPD and low TS survived longer than did patients with tumor having the other patterns of TS and DPD expression. Consistent with our findings, the combined evaluation of TS and DPD expression might predict the prognosis more accurately than using one marker in patients with prostate cancer. These data also reflect the report by Jakob *et al.*[11] that immunohistochemistry such as reverse transcriptase polymerase chain reaction is a suitable method to determine the correlation between TS and DPD expression and histopathologic tumor regression. However, the precise mechanisms for this relationship remain unclear at present. Additional studies are necessary to examine the mechanisms.

The benefit of hormonal therapy against advanced prostate cancer has been widely accepted. In addition, the antitumor effect is very high. Because TS is the key enzyme in the process of DNA synthesis, we believe it is reasonable to assume that this was the reason the TS expression was greater in the prostate cancer specimens

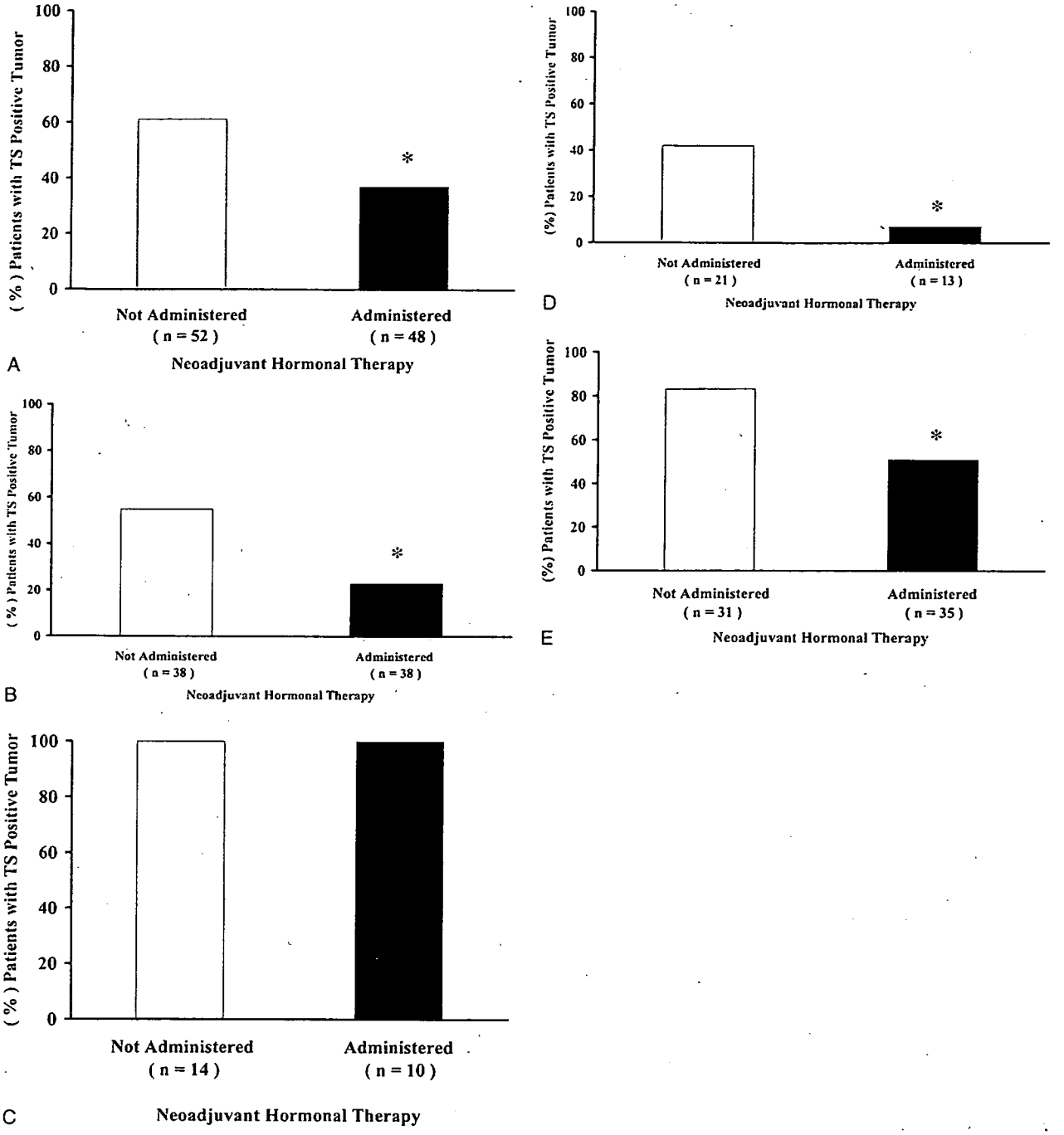


Figure 4. TS expression in prostate cancer with/without neoadjuvant hormonal therapy according to stage and Gleason grade of prostate cancer. TS expression in prostate cancer specimens evaluated by immunohistochemical assay as described in Material and Methods section. **(A)** $^{*}P = 0.0049$ versus not administered. **(B)** Patients with Stage T2 disease ($^{*}P = 0.0078$ versus not administered). **(C)** Patients with Stage T3 tumor. **(D)** Patients with Gleason score less than 7 tumor ($^{*}P = 0.0259$ versus not administered). **(E)** Patients with Gleason score 7 or greater tumor ($^{*}P = 0.0036$ versus not administered).

of the patients treated without neoadjuvant hormonal therapy than in those treated with surgery and neoadjuvant hormonal therapy. Furthermore, hormonal therapy might directly downregulate TS expression. A study by Ogasawara *et al.*[21] showed that in nude mouse MCF-7 breast cancer xenografts, tumor TS levels were reduced by treatment with a pure antiestrogen. Hung *et al.*[22] also reported that the Ki-67 proliferation index was significantly greater in TS-positive tumors than in TS-negative tumors, suggesting that TS-negative tumors might have a low rate of cell proliferation.

TS is a key enzyme for pyrimidine synthesis. DPD is an important pyrimidine salvage enzyme. No correlation was found between DPD and TS expression in colorectal cancer.[20,23] We reported that no correlation was found between the TS and DPD activity levels in bladder

carcinoma[12,24] or renal cell carcinoma.[13] Our data in prostate cancer are consistent with these results.

Immunohistochemical staining for TS and DPD predict the response to 5-FU.[10,11] Previous studies of several cancers have demonstrated that the TS expression level predicted the response to 5-FU-based chemotherapy.[4,5] Greater TS expression was accompanied by a greater response rate to 5-FU-containing chemotherapy. Most of the administrated 5-FU is degraded through the catabolic pathway with DPD.[18] DPD activity is highly associated with 5-FU pharmacokinetics.[25] The efficacy of 5-FU is related to the plasma level of this agent, which is inversely associated with the DPD activity level.[25] Our previous report demonstrated that primary cultured renal cell carcinoma cells with both high TS activity and low DPD activity were more sensitive to 5-FU than those with either low TS activity or high DPD activity.[26] These findings suggest that the TS and DPD expression levels in prostate cancer could be important predictive indicators for 5-FU efficacy. However, other factors might be more important, including the rate of degradation, carrier protein level, and so forth, although the principle TS and DPD expression levels might predict the response to 5-FU.

CONCLUSIONS

The results of our study have demonstrated that TS expression was significantly greater in the cancerous prostate and that positive TS expression was associated with a worse prognosis. These findings suggest that the assessment of TS expression might be useful in the management of prostate cancer. Because TS expression could be used as a prognostic parameter in patients with prostate cancer, an accurate prediction of prognosis might help to select patients for more intensive surgical, hormonal, or chemotherapeutic approaches, including 5-FU. Additional prospective studies are warranted to define the role of TS in selecting patients for adjuvant therapy for prostate cancer.

Acknowledgment. To Hal Gold for assistance in the preparation of this report.

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The significance of the expression of dihydropyrimidine dehydrogenase in prostate cancer

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Accepted for publication 11 September 2006

OBJECTIVE

To measure dihydropyrimidine dehydrogenase (DPD), an enzyme involved in the metabolism of 5-fluorouracil (5-FU), expression in prostate cancer and determine whether 5-chloro-2,4-dihydroxypyridine (CDHP), a potent inhibitor of DPD, enhances the antitumoral activity of 5-FU against prostate cancer.

PATIENTS, MATERIALS AND METHODS

In all, 44 prostate tissue specimens were obtained from men who had a radical prostatectomy alone for prostate cancer, and 38 specimens from men who had had neoadjuvant hormonal therapy. We analysed the cancerous tissue and normal prostate tissue for DPD expression using immunohistochemistry, and determined its prognostic significance. In cultured human prostate cancer lines (DU145 and LNCaP), we compared the cytotoxicity of 5-FU/CDHP with that of 5-FU alone. Finally, in experiments on immunodeficient mice, we studied the effect of oral administration of tegafur, a pro-drug for 5-FU, with or without CDHP on the growth of tumours introduced by injection of DU145 cells.

RESULTS

The expression of DPD was significantly higher in cancerous than normal prostate tissue; 36 of 44 (82%) specimens of prostate cancer expressed DPD, whereas only 25 of 44 (57%) specimens of normal prostate tissue expressed DPD. For men with prostate cancer who had radical prostatectomy alone, men with negative DPD expression tended to have a longer recurrence-free survival than those with positive expression; there were no recurrences in men with prostate cancer and negative DPD expression in the 5-year follow-up. DPD expression was significantly lower in men with prostate cancer who received neoadjuvant hormonal therapy. In vitro treatment of human prostate cancer cell lines with 5-FU/CDHP showed more cytotoxicity than with 5-FU treatment alone. Finally, DU145 tumours in mice treated with tegafur and CDHP were significantly smaller than in mice given tegafur alone.

CONCLUSION

The present study showed that DPD expression is elevated in prostate cancer, and indicate that DPD inhibitors might enhance the antitumour activity of 5-FU against prostate cancer.

KEYWORDS

DPD, prostate cancer, immunohistochemistry, 5-FU

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD), an important enzyme in the pyrimidine degradation pathway [1–6], is the rate-limiting enzyme responsible for converting thymine to dihydrothymine [7,8]. Early analyses of human tumour cell xenografts showed a wide range of DPD enzymatic activity among various solid and haematopoietic tumours [9–11]. As DPD is responsible for the degradation of 5-fluorouracil (5-FU), intratumoral DPD activity was investigated in clinical studies in patients with head-and-neck [12] and colorectal [13] cancers treated with 5-FU. DPD activity varies among individual tumours, and increased DPD activity is correlated with a poor clinical response to 5-FU-based chemotherapy [12,13]. Recently, immunohistochemistry was used to evaluate DPD protein expression *in situ* using paraffin-embedded blocks of specimens [14–16]. Like DPD activity, high levels of DPD expression were associated with a poor clinical response to 5-FU in nude mice with gastric cancer xenografts, and in patients with colorectal cancer [17].

To our knowledge, nothing is known about the expression of DPD in prostate cancer, or about its roles in prostate cancer. In the present study we investigated DPD expression by immunohistochemistry in prostate cancer tissue and in normal prostate tissue, and determined its prognostic significance. The effect of 5-chloro-2,4-dihydroxypyridine (CDHP), a DPD inhibitor, on 5-FU cytotoxicity against prostate cancer was also examined.

PATIENTS, MATERIALS AND METHODS

In all, 44 prostate cancer specimens with adjacent normal prostate tissue were obtained from the surgical pathological files of Kyoto Prefectural University of Medicine between 1997 and 2004. None of the patients had had preoperative androgen-deprivation therapy or radiotherapy. The cases were selected to represent the full spectrum of pathological stage and grade. The mean (range) age of the patients was 66 (53–75) years. The 2002 TNM system was used for pathological staging, and the final pathological stages included 30 cases of T2 and 14 of T3; 38 men with prostate cancer who had had neoadjuvant hormonal therapy (NHT) were also examined. The study was performed after approval by a local Human

Investigations Committee, and informed consent was obtained from each patient.

For immunohistochemistry, serial 5-μm sections were cut from formalin-fixed, paraffin wax-embedded slices of the specimens. The sections were de-waxed in xylene, rehydrated with graded alcohols, and antigen retrieved by microwave heating for 15 min. Endogenous peroxidase was blocked by incubating samples in 3% H_2O_2 in methanol. After washing three times in PBS, samples were placed in 10% normal equine serum (Cosmo Bio Co. Ltd, Tokyo, Japan) in PBS for 30 min to reduce nonspecific staining. The sections were then incubated with polyclonal antibody against DPD (Second Cancer Research Laboratory, Taiho Pharmaceutical Co. Ltd, Saitama, Japan) at 4 °C overnight. After washing three times in PBS, slides were incubated with Histofine Simplestain MAX-PO® (Nichirei Corporation Co. Ltd, Tokyo, Japan) at room temperature for 1 h. Immunohistochemical reactions were revealed with a solution of 3, 3′-diaminobenzidine tetrahydrochloride. The sections were then counted randomly at ×200.

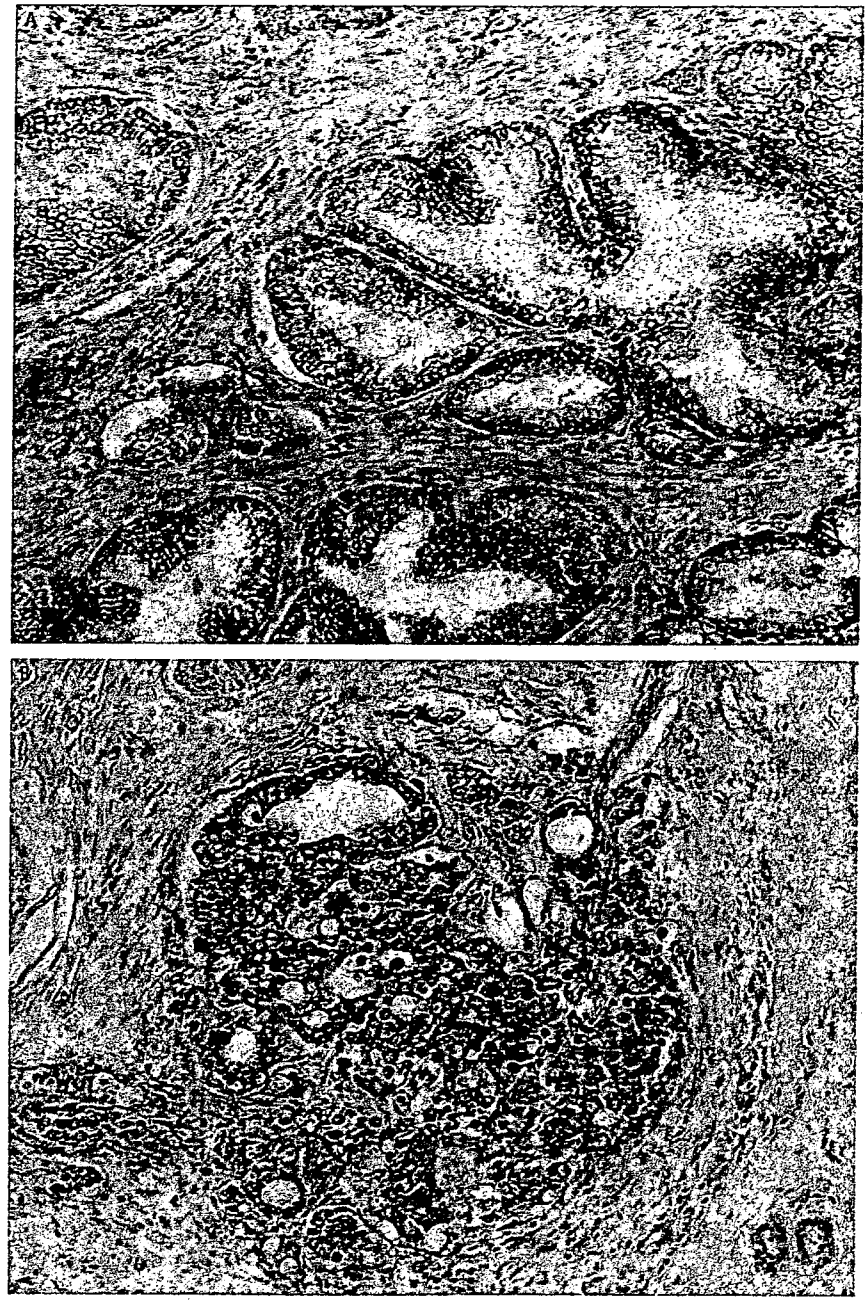
The intensity of DPD immunoreactivity was evaluated in normal prostate tissue and prostate cancer from the same slide for each case. The microscopic fields with the most immunoreactivity were chosen for analysis; [3] 1000 cells were analysed in each case. DPD was localized in the cytoplasm of both normal prostate tissue and prostate cancer cells. DPD expression was determined by a pathologist (Fig. 1); the intensity was graded from (–) to (+++). Samples with <10% positive cells were designated as negative (–), with 10–25% positive as +, 25–50% positive as ++, and >50% positive as +++.

The DU145 and LNCaP human prostate cancer cell lines were maintained in RPMI 1640 (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies Inc.) and 10% fetal bovine serum (Bio-cult, Glasgow, Scotland, UK) at 37 °C in a 5% CO_2 atmosphere.

Male severe combined immunodeficiency (SCID) mice (8–9 weeks of age) were purchased from CLEA Japan (Osaka, Japan), and housed in a specific pathogen-free animal facility. The mice were fed irradiated mouse chow and autoclaved water treated by reverse osmosis. The Committee for Animal Research, Kyoto Prefectural University of Medicine approved the experimental procedure.

5-FU (Lot no. 308033) was kindly supplied by Kyowa Hakkou Co. Ltd, Tokyo, Japan. Tegafur [1-(2-tetrahydrofuryl)-5-fluorouracil, FT], CDHP and potassium oxonate (OXO) were donated by Taiho Pharmaceutical Co. Ltd, Tokyo, Japan. FT, which is a prodrug of 5-FU, functions as an effector; OXO, which inhibits the conversion of 5-FU to 5-fluorouridine 5-monophosphate by

FIG. 1. Expression of DPD in prostate cancer and normal prostate tissue. Specimens were fixed in formalin, embedded in paraffin wax, and immunostained with DPD monoclonal antibody. Pictures were reduced from ×200. A, DPD-negative normal prostate tissue. B, Arrow *indicates DPD-positive prostate cancer.*



Cell type, n (%)	Staining intensity grade			
	–	+	++	+++
Normal prostate	19 (43)	16 (36)	9 (21)	0
Prostate cancer*	8 (18)	21 (48)	11 (25)	4 (9)

TABLE 1
Intensity of cells with DPD immunostaining in 44 RP specimens

**The staining intensity in prostate cancer was significantly higher than in normal prostate ($P = 0.02$, chi-square for independence test).*

orotate phosphoribosyltransferase, is mainly distributed in the gastrointestinal tract after oral administration in mice, and relieves the gastrointestinal tract toxicity induced by 5-FU.

For the cytotoxicity assay, a microculture tetrazolium dye (MTT) assay was used to determine cell lysis as previously [18]. Briefly, 100 μL of target cell suspension (2×10^4 cells) was added to each well of 96-well flat-bottom microtitre plates (Corning Glass Works, Corning, NY, USA), and each plate was incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, the supernatants were aspirated, and cells were washed three times with RPMI-1640 medium, and 200 μL of drug solution or medium (control) were distributed in the 96-well plates. Each plate was incubated for 24 h at 37 °C. After incubation, 200 μL of MTT working solution (5 mg/mL, Sigma Chemical Co., St. Louis, MO, USA) was added to each well, and the cultures were incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. The medium was removed from the wells and replaced with 100 μL of isopropanol (Sigma) supplemented with 0.05 M HCl. The absorbance of each well was measured with a microculture-plate reader (Immunoreader, Japan Intermed Co. Ltd, Tokyo, Japan) at 540 nm. The percentage cytotoxicity was calculated as [1 – (absorbance of experimental wells/absorbance of control wells)] × 100.

For the *in vivo* study with the DU145 cell line, 6×10^6 DU145 cells with a mixture of 50 μL Matrigel (Becton-Dickinson, NJ, USA) and 50 μL RPMI 1640 with no antibiotics or serum were injected s.c. into the right flanks of SCID mice; 13 days later, the DU145 tumour size was ≈120 mm^3. The mice were assigned to three groups of eight mice: control mice received saline orally; tumour-bearing mice were treated with FT/OXO, (8.3/8.3 mg/kg/day) or FT/CDHP/OXO (8.3/2.4/8.3 mg/kg/day) for 18 consecutive days. The tumours were measured at 3-day intervals until 18 days after the initial treatment; the diameter was scaled with a digital calliper and the volume calculated as $a \times b^2/2$, where a is the long diameter and b the short diameter.

Data were analysed by Student's t-test and chi-square test. Biochemical recurrence was defined as a postoperative serum PSA level of ≥ 0.1 ng/mL [19]. Postoperative recurrence-free survival rate was determined using the Kaplan–Meier method. All P-values were two-sided, and $P < 0.05$ was considered to indicate significance.

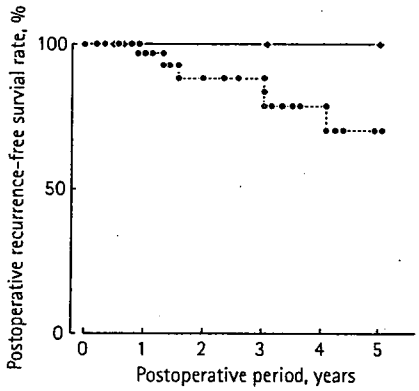
RESULTS

In specimens from men treated with radical prostatectomy (RP) alone, DPD expression was detected in 36 of 44 (82%) cancer samples ($P < 0.05$) compared to 25 of 44 (57%) specimens of normal prostate tissue. The staining was also significantly more intense in cancer cells than in normal prostate cells ($P = 0.02$; Table 1). However, there was no correlation between DPD expression and the stage/grade of the cancer (data not shown).

For men with prostate cancer who had RP alone, the recurrence-free survival was determined by Kaplan–Meier analysis. In the 5-year follow-up, men with negative DPD expression tended to have a longer recurrence-free survival than those with positive DPD expression, but the difference was not statistically significant ($P = 0.1$; Fig. 2). There were no recurrences in men with prostate cancer and negative DPD expression.

The expression of DPD in men with prostate cancer was higher in men treated with RP alone (82%; 36 of 44) than men treated with NHT (53%; 20 of 38; $P < 0.001$). For T2 cancer, DPD expression was lower in men treated with NHT (48%; 14 of 29) than with RP alone (80%, 24 of 30; $P < 0.001$). However, for stage T3 cancer, there was no significant difference in DPD expression between men treated with NHT (six of nine) or RP alone (12 of 14).

FIG. 2. Relationship between DPD expression and postoperative recurrence-free rate in men with prostate cancer. Postoperative recurrence-free rate of men with prostate cancer undergoing RP alone was determined by the Kaplan–Meier method. Men with prostate cancer were classed as those with positive DPD expression and those with negative expression. Men with prostate cancer with negative expression had a higher recurrence-free rate than those with positive expression in the 5-year follow-up ($P = 0.1$). Solid line, eight men with negative DPD expression; dashed line, men with positive DPD expression.



For well and moderately differentiated prostate cancer, DPD expression in men with RP alone (81%; 34 of 42) was significantly higher than in men treated with NHT (52%, 14 of 27; $P < 0.001$). However, for poorly differentiated prostate cancer, there was no significant difference in expression between men treated with RP alone (two of two) or NHT (five of 11).

Various inhibitors of DPD were developed in *in vitro* studies to increase the anticancer effects of 5-FU [20]; CDHP is a potent DPD inhibitor with no anticancer activity by itself. We examined whether CDHP enhanced the cytotoxic activity of 5-FU against prostate cancer cells *in vitro*. 5-FU/CDHP had a significant cytotoxic effect against both hormone-sensitive LNCaP (Fig. 3A) and hormone-resistant DU145 (Fig. 3B) cells compared with 5-FU alone.

FT/OXO and FT/CDHP/OXO were orally administered for 18 days to SCID mice bearing the DU145 cancer. No mice had died by 18 days. On day 18, mice given FT/CDHP/OXO

had significantly smaller tumours than mice given PBS ($P = 0.01$) or FT/OXO ($P = 0.03$; Fig. 4).

DISCUSSION

This is the first report that DPD expression is higher in prostate cancer than in normal prostate tissue; furthermore, men with prostate cancer and positive DPD expression had a higher recurrence rate than men with negative DPD expression during the 5-year follow-up. Thus, positive DPD expression might be associated with a worse prognosis, and DPD might be a molecular therapeutic target in prostate cancer.

The present data indicate that DPD expression in prostate cancer is significantly higher than in normal prostate tissue; ≈43% of normal prostate tissue lacked DPD expression, while most prostate cancer specimens expressed DPD. We previously reported that DPD activity in bladder cancer tissue is twice that in normal bladder tissue [3]. Horiguchi et al. [15] reported that 59 of 119 (50%) patients had positive immunostaining for DPD in breast cancer, and clarified the prognostic significance of the DPD expression in breast cancer. DPD expression, as estimated by immunohistochemical analysis in the preoperative biopsy, was comparable to that in resected gastric carcinoma [21]. The level of DPD activity in malignant cell lines was related to malignant behaviour [22]. These studies indicate that the expression of DPD in various cancers is significantly higher than in normal tissue.

Sensitivity to 5-FU can be enhanced by using a DPD inhibitor like CDHP [20], and DPD inhibition is a major goal in the strategy for the development of 5-FU treatment. Several authors reported that DPD activity and DPD mRNA expression are inversely correlated with chemosensitivity to 5-FU *in vitro* and in patients with cancer. Thus, DPD is not only a key modulator of 5-FU pharmacokinetics, but also a good predictor of responsiveness to 5-FU [20]. In the present study, CDHP enhanced 5-FU cytotoxicity in prostate cancer cells *in vitro*, and oral administration of CDHP enhanced the antitumour activity of 5-FU against prostate cancer cells in SCID mice. DPD appears to be important in regulating 5-FU sensitivity in prostate cancer. Accordingly, we consider it valuable to establish a simple and reliable method to assess DPD expression in prostate cancer. In addition, combined therapy with 5-FU and DPD inhibitors might be effective against prostate cancer.

In the present study, DPD expression was significantly higher in prostate cancer, and positive-DPD expression was associated with a worse prognosis. These findings suggest that assessing DPD expression might be useful for the management of prostate cancer. As DPD expression might be used as a prognostic indicator in men with prostate

*FIG. 3. Enhancement of the sensitivity of prostate cancer cells to 5-FU by CDHP. LNCaP (A) and DU145 (B) cells were treated with 5-FU (1–100 μM) in combination with CDHP (100 μM) for 24 h and the cytotoxicity was assessed by a 1-day MTT assay. Results from three different experiments are expressed as the mean (SD). *P < 0.05 vs 5-FU alone. White bar, 5-FU alone; black bar, 5-FU + CDHP (100 μM).*

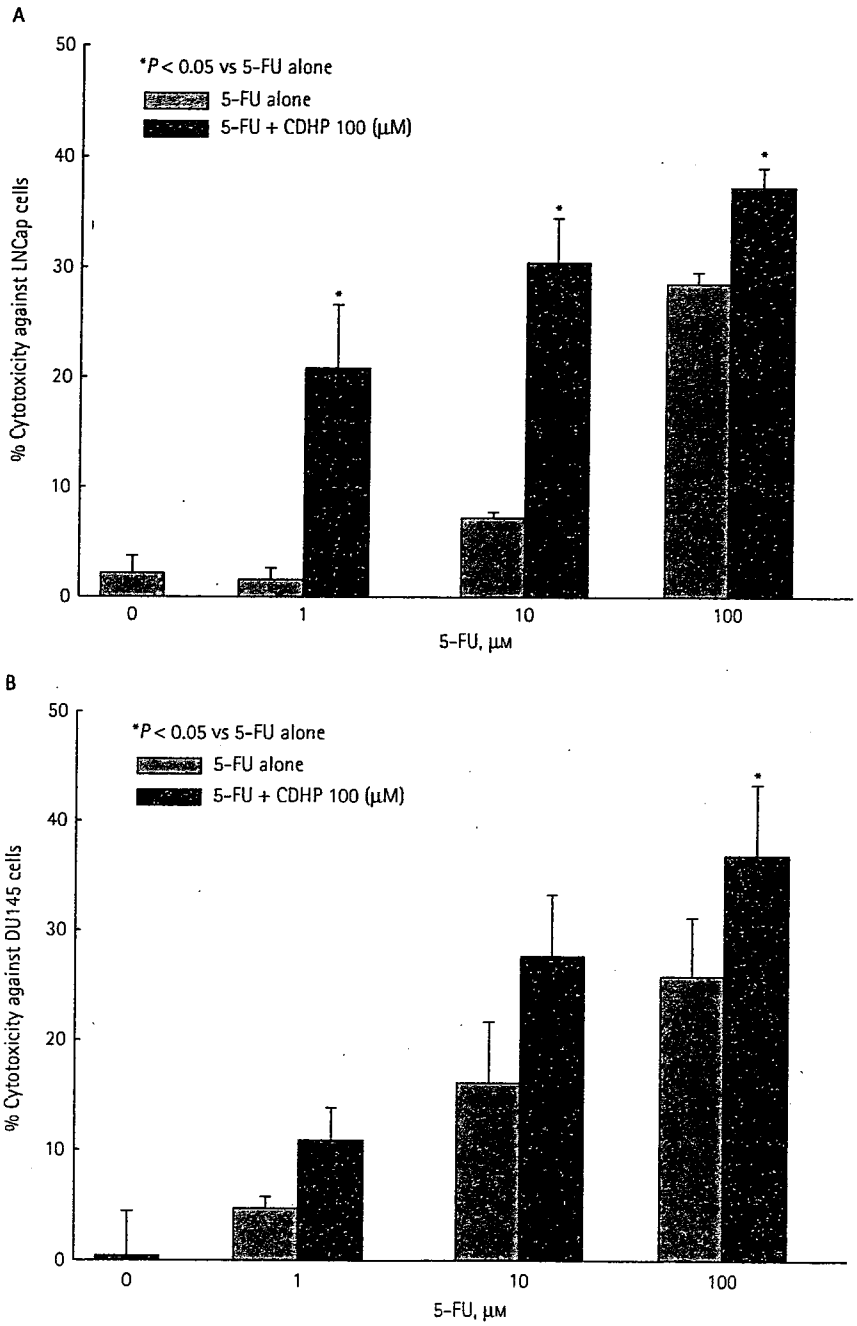
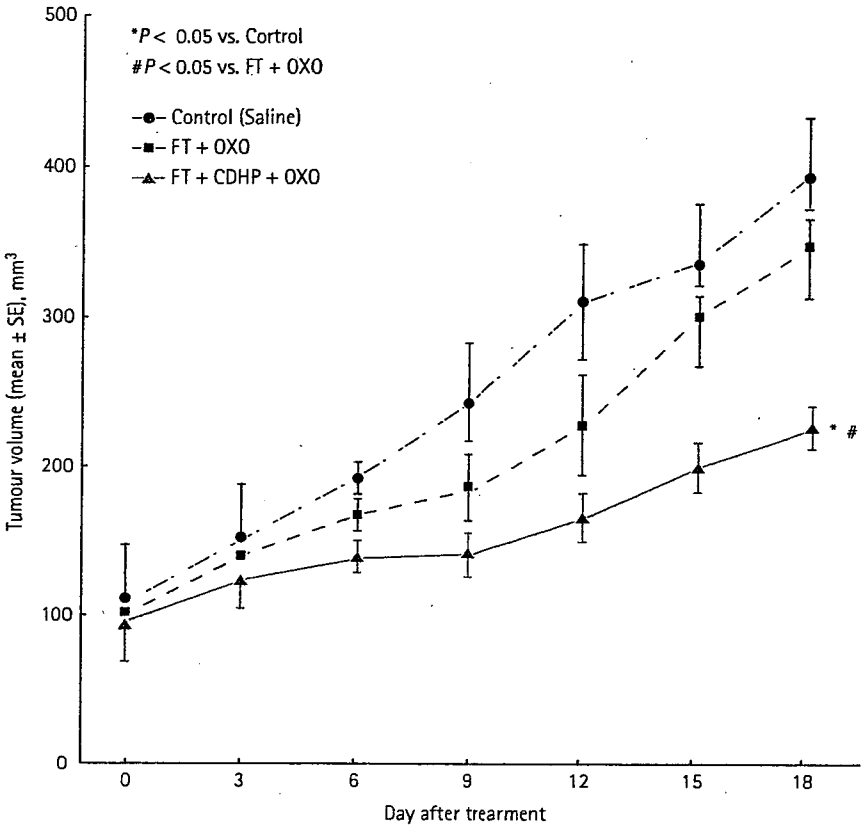


FIG. 4. In vivo *antitumoral effects of FT/CDHP/OXO on DU145 cells. Mice bearing tumour with a starting volume of 120 mm³ were treated with oral administration of saline, FT/OXO (8.3/8.3 mg/kg), or FT/CDHP/OXO (8.3/2.4/8.3 mg/kg) daily; eight mice per group.* *P < 0.05 vs control, #P < 0.05 vs FT/OXO.



cancer, the accurate prognosis might help doctors to decide upon more intensive therapeutic approaches in combination with DPD inhibitors. However, further studies are required for confirmation.

ACKNOWLEDGEMENTS

This work was supported in part by Grant-in-Aids from the Japanese Ministry of Education, Culture, Sports, Science and Technology (NO. 18390439 and no. 18659479). We acknowledge the technical assistance of Dr Masakazu Fukushima (Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd. Saitama, Japan) and Mr Hal Gold for assistance in the preparation of this manuscript.

CONFLICT OF INTEREST

None declared.

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