External Validation of a Nomogram Predicting the Probability of Prostate Cancer Gleason Sum Upgrading Between Biopsy and Radical Prostatectomy Pathology Among Japanese Patients

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OBJECTIVES To explore the rate of upgrading in a contemporary cohort from 2 Japanese institutions, and evaluating the predictive accuracy of the nomogram when applied to patients, regardless of race. Previous reports have indicated that a maximum of 43% of men with prostate cancer will show an upgraded Gleason score from biopsy to radical prostatectomy (RP). A preparative nomogram was developed at the University of Hamburg to predict the probability of upgrading from biopsy to RP specimen.

METHODS Clinical and pathologic data of 503 patients from 2 Japanese institutions were supplied for validation. Nomogram-predicted probabilities of upgrading from biopsy to RP specimen were compared with actual rate of upgrading. The area under the receiver operating characteristic curve (AUC) was calculated for all patients. Calibration of the nomogram was achieved by comparing the predicted upgrading rate with that of an ideal nomogram.

RESULTS Gleason sum upgrading was recorded in 29.8% of patients at RP. Accuracy of the nomogram was 79.2% (confidence interval, 75.1%-83.2%). Overall AUC was 0.79 when applied to the validation dataset, with individual institutional AUCs ranging from 0.79-0.80. Nomogram predictions of upgrading were not within 10% of an ideal nomogram.

CONCLUSIONS Gleason sum upgrading between biopsy and final pathology represents an important consideration in treatment decision-making, and nearly one third of patients with prostate cancer will be upgraded. The Hamburg nomogram seems to provide reasonably accurate predictions regardless of minor variations in pathologic assessment, but is not necessarily so accurate when applied to Japanese patient population. UROLOGY xx: xxx, xxxx. © 2009 Elsevier Inc.

Pretreatment prostate-specific antigen (PSA) levels, Gleason score, and pathologic stage are generally recognized as significant predictors of biochemical recurrence in patients with clinically localized prostate cancer treated with radical prostatectomy (RP). A finding of high-grade disease in RP specimens is an adverse prognostic factor, and such tumors are significantly more likely to progress than organ-confined cancers. Pathologic Gleason sum represents a better predictor of biochemical recurrence than the biopsy Gleason sum.[1] A high RP Gleason sum is associated with a higher rate of biochemical recurrence and worse prostate cancer-specific survival.[2,3]

Any upgrade of Gleason sum from that of the biopsy to the final pathologic specimen can alter the treatment options.[4,5] In surgical candidates, grade changes from a Gleason sum of 6 to ≥7 increases the rate of extracapsular extension (ECE) and should prompt the clinician to consider a treatment modality in which ECE can be effectively addressed. Some recent reports[6-8] have suggested that a maximum of 43% of men with low-grade

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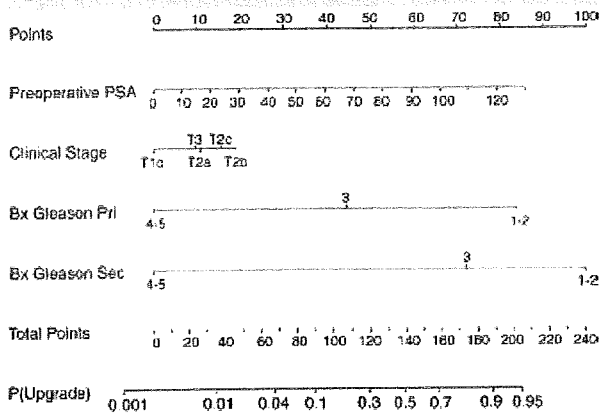

Figure 1. Nomogram based on 2982 patients treated at the University of Hamburg, for predicting Gleason sum upgrading between biopsy and radical prostatectomy. Data adapted from Chun et al.[9] PSA indicates prostate-specific antigen (ng/mL); BX Gleason PRI = primary biopsy Gleason score; BX Gleason Sec = Secondary biopsy Gleason score; P (Upgrade) = probability of upgrade from biopsy Gleason sum.

prostate cancer at biopsy will be diagnosed with high-grade prostate cancer at RP.

Three models were developed to predict the probability of Gleason sum upgrading (GSU).[9-11] One was externally validated and yielded a concordance index of 0.5.[11] The second model achieved 71% accuracy in internal validation. However, it relied on 9 predictor variable, some of which are not routinely recorded in charts of the patients.[10] Chun et al[9] reported a prognostic model (1) that was 80.4% accurate in predicting the probability of biopsy GSU. Currently, this represents the only available and highly accurate clinical aid capable of predicting pathologic GSU. However, this accuracy estimate was not confirmed in an independent external population, which prompted vivid criticisms that were founded on lack of formal external validation. On the basis of importance of the concept of GSU in decision-making for prostate cancer, we decided to perform a formal external validation using a fully independent dataset in a contemporary cohort of 2 Japanese institutions.

MATERIAL AND METHODS

Validation data representing men treated with RP were obtained from 2 Japanese institutions: the Department of Urology in the Graduate School of Medicine at Chiba University, Chiba (n = 214); and the Division of Urology at Chiba Cancer Center, Chiba (n = 295). For prediction, this nomogram[9] requires pretreatment PSA level, clinical stage, and biopsy Gleason sum (Fig. 1).

A total of 509 patients met the derivation criteria. All men had biopsy-confirmed, clinically localized prostate cancer and underwent RP between February 1997 and January 2008. Of these, 6 patients were excluded because of missing data. Analyses targeted 503 evaluable patients assessed with ≥6 biopsy cores. Patients treated with neoadjuvant hormonal therapy were excluded because the nomogram is not applicable in these men.

Clinical stage was assigned by the attending urologist according to the 2002 tumor-node-metastasis system. Under transrectal ultrasound guidance, 6-16 needle cores were obtained. Pretreatment PSA levels were measured before a digital rectal examination and transrectal ultrasound. Biopsy Gleason sum was assigned by pathologists from each center. All RP specimens were processed according to the Stanford protocol and were graded according to the Gleason system.[12]

The probabilities were derived by plugging in each individual patient into the nomogram by a web-based format. Accuracy of the nomogram was quantified using the receiver operating characteristics curve. To determine the nomogram-predicted probability of GSU, we applied the nomogram described by Chun et al (Fig. 1) to all 503 observations. Accuracy of the nomogram was then quantified using the area under the curve (AUC) for external validation. The extent of over- or underestimation relative to the observed rate of upgrading was explored graphically using nonparametric Loess smoothing plots. All tests were two-sided, with a significance level set at $P < .05$.

RESULTS

Table 1 lists the clinical and pathologic characteristics of patients included in this validation dataset and Hamburg nomogram development cohort,[9] and data were stratified between the entire cohort and for participating institutions. Pretreatment PSA levels were 2.6-80 ng/mL, and PSA levels >20 ng/mL were recorded in 82 patients (16.3%), as compared with ≤10 ng/mL in 258 men (51.3%). Clinical stages T1c and T2 were recorded in 452 patients (89.9%). Among all men, 323 (64.2%) had a biopsy Gleason sum of 6 or 7. Furthermore, we compared the population used by Hamburg nomogram development and the current study, with regard to predictor variables. It was found that the clinical stage, biopsy, and pathologic Gleason patterns of Japanese men were significantly higher than those of German patients. The PSA values, biopsy, and pathologic Gleason sums of the current study were also higher, but not significantly.

Table 2 shows the concordance between biopsy and prostatectomy Gleason sum. In all patients, concordance between biopsy and RP Gleason sum was recorded in 232 (46.1%). Overall upgrading was recorded in 150 men (29.8%), whereas 121 (24.1%) were downgraded. Gleason sum decreased from ≥8 to ≤7 in 57 (11.3%), and from 7 to ≤6 in 34 (6.8%) men. Stratified according to institutions, agreement between Gleason biopsy and final pathology was more frequent in the Chiba University dataset (103 men, 49.5%) than in that from Chiba Cancer Center (129, 43.7%). Interestingly, upgrading was more frequent for Chiba University (68 men, 32.7%) than for Chiba Cancer Center (82, 27.8%). For the entire cohort, up- and downgrading were reported in 150 (29.8%) and 121 (24.1%), respectively (Table 2).

Figure 2 illustrates how predictions of the nomogram compared with the actual probabilities for the 503 men. The x axis shows the prediction calculated using the nomogram, and the y axis gives observed rates of GSU for

Table 1. Descriptive characteristics of the entire cohort and subgroups according to institutions

Variable	Entire Cohort	Chiba University	Chiba Cancer Center	Hamburg Nomogram, Cohort[9]
No. patients	503	208	295	2982
Age (y)				
Mean	66.16501	65.38462	66.71525	
SD	5.322527	5.338417	5.250877	
Median	66	66	67	
Min	49	49	52	
Max	78	77	78	
PSA				
Mean	13.72459	12.82548	14.35854	9.6
SD	12.2115	11.74656	12.50982	
Median	9.79	9.005	10.116	7.0
Min	2.588	3.57	2.588	0
Max	79.71	74	79.71	125
Clinical stage (%)				
T1c	199 (39.6)	123 (59.1)	198 (67.1)	1951 (65.4)
T2a	151 (30.0)	29 (13.9)	122 (41.4)	493 (16.5)
T2b	19 (3.8)	19 (9.1)	0 (0.0)	349 (11.7)
T2c	83 (16.5)	18 (8.7)	65 (22.0)	108 (3.6)
T3	51 (10.1)	19 (9.1)	32 (10.8)	81 (2.7)
Biopsy Gleason primary (%)				
≤3	345 (68.6)	149 (71.6)	196 (66.4)	2667 (89.5)
4	132 (26.2)	50 (24.0)	82 (27.8)	310 (10.4)
5	26 (5.2)	9 (4.3)	17 (5.8)	5 (0.2)
Biopsy Gleason secondary (%)				
≤3	276 (54.9)	127 (61.0)	149 (50.5)	2209 (74.1)
4	176 (35.0)	69 (33.2)	107 (36.3)	742 (24.9)
5	51 (10.1)	12 (5.8)	39 (13.2)	31 (1.0)
Biopsy Gleason sum (%)				
≤6	208 (41.3)	103 (49.6)	105 (35.6)	1993 (66.8)
7	193 (38.4)	69 (33.2)	124 (42.0)	887 (29.7)
8	49 (9.7)	22 (10.6)	27 (9.2)	73 (2.4)
9	46 (9.1)	11 (5.3)	35 (11.9)	29 (1.0)
10	7 (1.4)	3 (1.4)	4 (1.4)	0 (0.0)
Pathological Gleason primary (%)				
≤3	331 (65.9)	140 (67.3)	191 (64.7)	2608 (87.5)
4	149 (29.6)	63 (30.3)	86 (29.2)	368 (12.3)
5	23 (4.6)	5 (2.4)	18 (6.1)	6 (0.2)
Pathologic Gleason secondary (%)				
≤3	258 (51.3)	117 (56.3)	141 (47.8)	1719 (57.6)
4	195 (38.8)	79 (38.0)	116 (39.3)	1223 (41.0)
5	50 (9.9)	12 (5.8)	38 (12.9)	40 (1.3)
Pathological Gleason sum (%)				
≤6	153 (30.4)	72 (34.6)	81 (27.4)	1397 (46.8)
	270 (53.7)	112 (53.8)	158 (53.6)	1527 (51.2)
	24 (4.8)	9 (4.3)	15 (5.1)	19 (0.6)
	55 (10.9)	15 (7.2)	40 (13.6)	39 (1.3)
	1 (0.2)	0 (0.0)	1 (0.3)	0 (0.0)
Gleason sum (%)	150 (29.8)	68 (32.7)	82 (27.8)	875 (29.3)

validation cohort. Accuracy of the nomo- .2% (confidence interval, 75.1%-83.2%). JC was 0.79 when applied to the validation dataset, with individual institutional AUCs ranging from 0.79-0.80.

The dashed line represents the performance of an ideal nomogram, where predicted outcome would correspond perfectly with actual outcome. The performance of Hamburg nomogram is plotted as the solid line. The dotted lines represent a 10% margin of error, which was speculated in the original nomogram publication.[9] The solid line is not close to the dashed line of the ideal nomogram and is not always within the 10% margin of error. The correspondence seen between actual and ideal nomogram predictions does not suggest good calibration of the nomogram in the validation cohort. For predicted values between 10% and 40%, the nomogram tends to underestimate the rate of GSU. Conversely, for predicted probabilities >65%, the nomogram tends to overestimate the true rate of GSU.

Table 2. Upgrading, downgrading, and agreement between biopsy and RP Gleason sum in 503 patients

Pathologic Gleason Sum	Biopsy Gleason Sum						Upgrade			Agree	Downgrade		
	≤6	7	8	9	10	Total	Up	Up 1	Up 2+		Down	Down 1	Down 2+
≤6	116	34	2	1	0	153	21	17	4	71	61	35	26
7	84	132	31	21	2	270	84	63	21	132	54	31	23
8	6	11	6	1	0	24	17	11	6	6	1	1	0
9	2	15	10	23	5	55	27	10	17	23	5	5	0
10	0	1	0	0	0	1	1	0	1	0	0	0	0
Total	208	193	49	46	7	503	150 (29.8%)	101 (20.1%)	49 (9.7%)	232 (46.1%)	121 (24.1%)	72 (14.3%)	49 (9.7%)

COMMENT

Pretreatment PSA level, Gleason score, and pathologic stage are generally recognized as significant predictors of biochemical recurrence in patients with clinically localized prostate cancer treated by RP.[13] The finding of high-grade disease in RP specimens is an adverse prognostic factor, and such tumors are significantly more likely to progress than organ-confined cancers. In addition, this finding is associated with a greater risk of positive surgical margins, further decreasing the likelihood of long-term cancer control. Consequently, determining whether a patient has high-grade disease is important for treatment selection and prognosis.[14]

Biopsy upgrading has important clinical implications in terms of watchful waiting, surgery, and radiotherapy candidates.[10,15,16] Most reported biopsy Gleason sums consist of either 6 or 7; these Gleason sums are at greatest risk of being upgraded. However, to date no tools have been available for reliably and accurately predicting this phenomenon. To address this absence, Chun et al[9] successfully developed and validated a model predicting GSU from biopsy to final pathology using clinical variables (PSA level, clinical stage, and biopsy Gleason sum). Their model relies on 3 readily available clinical variables, all of which are significant uni- and multivariate predictors of biopsy GSU.

Moreover, previous reports have indicated that with more extended biopsy schemes, the risk of upgrading decreases[15,17] because of higher sampling density and more accurate evaluation of the pathologic biopsy. In Hamburg nomogram development cohort, of all 2982 men, 2363 (79.2%) had 6, 61 (2.0%) had 8, 538 (18.0%) had 10, and finally 20 (0.7%) had 12-core biopsies taken.[9] Extended biopsy schemes (≥10 cores) might affect the rate of and ability to predict biopsy GSU. As a result, ≥14 needle cores are obtained in our institutions.[18] The present study included old data in which patients were exposed to <10 cores, so 6-16 needle cores were obtained. The differences in biopsy schemes between the original cohort from Germany and the current study may represent an important limitation. The relationship between the biopsy and RP Gleason sum would be drastically affected by the extent of gland sampling.

The present findings are important as a first substantial depiction of the rate of GSU in a Japanese contemporary cohort. The present cohort of 503 patients showed an overall upgrading rate of 29.8%, comparable to the 29.3% described by Chun et al. Close agreement between the present results and those data can be explained by the similar extent of gland sampling in both studies. Other explanations might include similar patient characteristics, such as PSA level, clinical stage, and distribution of biopsy Gleason sum. Several applications of the present findings can be considered. The choice of interstitial brachytherapy, for example, might be reconsidered in men who are at greater risk of biopsy GSU. Similarly, neoadjuvant hormonal therapy might be con-

sidered if radiotherapy is contemplated. Finally, among surgical candidates, the risk of biopsy GSU might contribute to different considerations regarding the extent of neurovascular bundle resection and the implications of positive surgical margins.

We are the first to recommend the use of multivariate models to predict GSU between biopsy and RP in Japanese population. To date, no other models capable of accurately predicting the rate of upgrading are available. Consequently, despite the limitations, such as imperfect predictions, this model represents the only alternative to clinical ratings of the probability of biopsy GSU. We have, therefore, tested the performance of the nomogram in an external dataset. Overall, AUC was 0.79 when applied to the validation dataset. In the initial derivation study of the Hamburg nomogram, Chun et al[9] validated the predictive tool, using a separate sample of 2982 men operated on by 5 separate surgeons from the same institution. With this cohort, the nomogram performed with high predictive accuracy (AUC, 0.804). Considering the homogeneity and similarity of this internal validation cohort as well as similarities in staging, diagnosis, and treatment processes, an assumption that predictive accuracy would decrease when applied to a multi-institutional validation dataset is unsurprising. Nonetheless, the overall accuracy of the nomogram in the current study is virtually the same as in the original report. The observed AUC of 0.79 still represents a high level of predictive accuracy. Individual treatment centers in this study differed with respect to patient selection, ECE measurement, and follow-up assessment. Furthermore, no centralized review of pathology was performed. For the purposes of nomogram validation, such heterogeneity is desirable to gain insights into how the nomogram will perform across varied settings.[19] The nomogram was consistently accurate at both centers, with a range in AUC from 0.79-0.80. Consequently, the achieved results in this study are of importance and demonstrate the strength of the initial tool. Further assessment in a community hospital setting might be valuable. As nomograms use different subsets of patients and require end points to be coded differently, comparison on matching datasets is difficult. The Hamburg nomogram thus seems to provide reasonably accurate predictions, regardless of minor variations in pathologic assessment.

There are clear limitations in the present study. We included systematic sextant and 14-16 core biopsy data in the cohort, but with the current data the difference in rate of upgrading was not significant between these biopsy regimens.[20] However, biopsy schemes that rely on taking even more cores might be associated with a lower rate of biopsy GSU. Besides the small population size, the level of experience of the pathologists could also contribute to the findings. Our institutions both represent centers with a large surgical volume, and rates of upgrading might, therefore, be lower than in smaller volume hospitals. Finally, accuracy of the model could potentially be improved by integrating additional predictor variables, such as the level of expertise of the pathologist, or existing biomarkers.[21] Despite these limitations, this model represents an important contribution, and we examined the rate of GSU between biopsy and final pathology.

Prostate cancer is one of the most common cancers among Western population, and incidence is increasing in Asia, although considerable differences in incidence and biological aggressiveness remain between Western and Asian population.[22] Epidemiologic and genetic differences in prostate cancers exist between patients in Japan and the United States, and p53 gene mutational analysis, which often provides information about etiologic factors, has revealed clear differences in p53 gene mutational spectra between Japanese and Western cases.[23] Differences in hormone levels in various racial and/or ethnic groups have been suggested to account for part of the differences in prostate cancer risk. Racial and/or ethnic differences in the intraprostatic testosterone and/or dihydrotestosterone conversion ratio would provide important support for the hypothesis that differences in the enzymatic activity of 5a-reductase within the prostate gland can explain most of the racial and/or ethnic differences in prostate cancer risk.[24-26] Clinical and pathologic differences on the basis of ethnicity between prostate cancer men do indeed exist. In the present study it was found that the clinical stage, biopsy, and pathologic Gleason patterns of Japanese men were significantly higher than those of German patients. The PSA values, biopsy, and pathologic Gleason sums of the current study were also higher, but not significantly. These findings are thought to be also reflected by the fact that considerable differences in degree of grade and stage migration remain between Western and Asian population.

Despite the good performance demonstrated in terms of AUC, predicted GSU probability from the nomogram was not within 10% of the observed GSU probability throughout the spectrum of predictions (Fig. 2). For low predicted values, especially between predicted values of 10% and 40%, the nomogram tends to underestimate the rate of GSU. The maximum rate of under-prediction seems to apply to men with predicted probabilities between 20% and 35%, but it remains within the 15% error range. By contrast, the assessment of the calibration plot also implies that the nomogram-predicted probabilities of >65% systematically overestimate the true rate of GSU. In consequence, it should be suggested either it is inappropriate to use the nomogram, or the predictions need to be interpreted as excessively high when predicting patients with a high likelihood of GSU. Accuracy in these men is important and affects treatment selection, because they are potentially considered to show a higher rate of biochemical recurrence and worse prostate cancer-specific survival. However, the decision of what level of risk is required for more aggressive therapy remains controversial. Perhaps on the basis of practical consider-

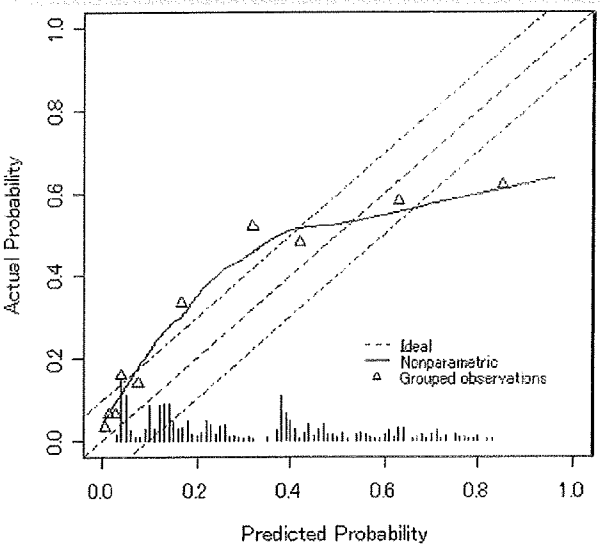

Figure 2. Calibration plot of external validation cohort. The x axis shows the prediction calculated using the nomogram, and the y axis gives observed rates of Gleason sum upgrading for patients in the validation cohort. Dashed line indicates reference line, where an ideal nomogram would lie. Solid line indicates performance of the Hamburg nomogram applied to the validation cohort. The solid line is not close to the dashed line of the ideal nomogram and is not always within the 10% margin of error.

ations, a probability of GSU of 65% vs 80% makes no differences, and treatment considerations might be based on the assumption that GSU is highly expected. It is also possible that the population differences described earlier might account for these observed discrepancies between predicted and observed tools.

Given these differences, development of a nomogram predicting the probability of biopsy GSU in a large cohort among Japanese patients seems essential. In the present no alternative to the existing tool has been available for reliably and accurately predicting GSU. Further study, with large clinical trials, is needed to develop the nomogram. Until then, we suggest that discussion between patient and physician regarding benefits and consequences should be centered around risk estimations.

King[7] and King and Long[6] defined significant GSU as a Gleason sum increase either from ≤ 6 to ≥ 7 or from 7 to ≥ 8 between the biopsy and RP specimens. They distinguished between any upgrading and significant upgrading and suggest that significant upgrading represents a clinically meaningful entity. It would be extremely more clinically meaningful to predict the rate of significant upgrading because these 3 categories represent pathologically and clinically different diseases. In view of the concept, we should create a new prediction tool, developed on a modern, Japanese-only cohort, aimed at predicting significant upgrading in future.

In conclusion, GSU between biopsy and final pathology represents an important consideration in treatment decision-making, even in most contemporary patients. Nearly one-third of patients with prostate cancer will be upgraded. The Hamburg nomogram seems to provide reasonably accurate predictions regardless of minor variations in pathological assessment, but cannot necessarily be considered accurate when applied to Japanese patient population.

CONCLUSIONS

The present findings confirmed the accuracy of the GSU nomogram and provide information about potential differences that might exist between expected and observed rates of GSU. The Hamburg nomogram provides reasonably accurate predictions regardless of minor variations in pathologic assessment, but cannot necessarily be considered accurate when applied to Japanese patient population. Our results suggest that development of a nomogram predicting the probability of biopsy GSU in a large multi-institutional cohort among Japanese patients is essential.

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Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells

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Abstract

Protein tyrosine kinase plays a central role in the proliferation and differentiation of various types of cells. One of these protein kinases, Tyk2, a member of the Jak family kinases, is known to play important roles in receptor signal transduction by interferons, interleukins, growth factors, and other hormones. In the present study, we investigated Tyk2 expression and its role in the growth and invasiveness of human prostate cancer cells. We used a small interfering RNA targeting Tyk2 and an inhibitor of Tyk2, tyrphostin A1, to suppress the expression and signaling of Tyk2 in prostate cancer cells. We detected mRNAs for Jak family kinases in prostate cancer cell lines by RT-PCR and Tyk2 protein in human prostate cancer specimens by immunohistochemistry. Inhibition of Tyk2 signaling resulted in attenuation of the urokinase-type plasminogen activator-enhanced invasiveness of prostate cancer cells *in vitro* without affecting the cellular growth rate. These results suggest that Tyk2 signaling in prostate cancer cells facilitate invasion of these cells, and interference with this signaling may be a potential therapeutic pathway.

Keywords: Tyk2; Jak; Prostate cancer; Invasion; Metastasis

Prostate cancer arises as a consequence of an imbalance between cell division and differentiation. The proliferation, differentiation, growth, and apoptosis of normal and malignant cells are regulated by many different cytokines and growth factors. Protein tyrosine kinases (PTKs) play a central role in the proliferation and differentiation of various types of cells. They participate in the cellular responses to growth factors, and activation of their protein kinase activity is critical for the transmission of mitogenic signals. In a previous study, to explore the function of PTKs in the developing prostate gland, we screened for PTKs expressed in CD44-positive cells from the developing mouse prostate. CD44 is a cell surface glycoprotein receptor and this signaling regulates several important biologic processes including lymphocyte homing and activation, hematopoiesis, and tumor progression and metastasis [1]. In addition, CD44 is expressed during mouse prostate development but not in the adult prostate. Treatment with neutralizing antibodies to CD44 inhibits androgen-stimulated ductal branching morphogenesis in serum-free organ cultures of the mouse prostate [2]. Therefore, CD44 is one of the markers of early progenitor cells in prostate tissues. Using CD44 as a cell surface marker, we isolated several PTK genes including Tyk2 from CD44-positive prostate cells [3].

To date, four mammalian members of the Jak family have been identified, namely, Tyk2, Jak1, Jak2, and Jak3. Previous reports from other laboratories have also demonstrated that Jaks are expressed in a human prostate cancer xenograft model and in bone marrow metastases [4,5]. Extensive studies over the last few years have suggested

Abbreviations: Jak, Janus kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; SH2, Src homology 2; Stat, signal transducers and activators of transcription.

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that Jak kinases play important roles in the responses to interferons, interleukins, growth factors, hormones, and urokinase-type plasminogen activator (uPA), also known as urokinase [6,7]. Activation of Jaks leads to the tyrosine phosphorylation of receptors, producing docking sites for various SH2-containing signaling molecules including Stat proteins [8]. It is well documented that Stats are over-activated in some malignancies. For example, Stat3 activity is elevated in prostate cancer [9]. Accumulating evidence for constitutive activation of various Stats and other oncoproteins in different cancers strongly suggests that Jak kinases play critical roles in the pathogenesis of many human neoplastic diseases [6].

In this study, we examined the expression and biological significance of Tyk2 in prostate cancer. We show that Tyk2 is involved in uPA-induced cell invasion, which is a measure of the malignant potential of prostate cancer cells. Blockade of Tyk2 signaling by a small interfering RNA (siRNA) or by the PTK inhibitor tyrphostin A1 significantly suppresses the invasiveness of human prostate cancer cells into Matrigel. Our results demonstrate that activation of the Tyk2 signaling pathway is important for the enhancement of prostate cancer cell invasiveness by uPA. Thus, the Tyk2 signaling pathway may be a worthwhile target for therapeutic intervention in prostate cancer.

Materials and methods

Cell culture and reagents. Three human prostate cancer cell lines LNCaP, PC-3, DU145, and TSU-Pr1 (bladder cancer cell line) and MCF-7 (breast cancer cell line) were used in this study. The cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell growth was assessed using a colorimetric proliferation assay employing the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H* tetrazolium (MTS). Each day, MTS was added, and the absorbance at 490 nm was measured on microplate reader after a 60-min incubation at 37 °C. The siRNA duplex targeting Tyk2 and a control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For transfection with siRNAs, DU145 cells were placed in Opti-MEM I (Invitrogen, Carlsbad, CA) and then transfected using oligofectamine (Invitrogen). Tyrphostin A1 (Sigma–Aldrich, St. Louis, MI), an inhibitor of Tyk2 PTK activity, was used at 100 μM, and the effect of uPA (R&D Systems, Minneapolis, MN) was examined at a concentration of 5 nM.

RT-PCR analyses. For RNA expression analysis, total RNA was extracted from cells using RNA Bee (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. The RNA was then treated with DNAse I to remove contaminating DNA and then reverse transcribed using an oligo-dT primer and Super-Scriptβ reverse transcriptase (Invitrogen) in a volume of 25 μl. The primer sequences were as follows: human Jak1, 5′-AAGTGATGTCCTTACCACA-3′ and 5′-AGCAGCCACAC TCAGGTTCT-3′; human Jak2, 5′-GAGCCTATCGGCATGGAATA-3′ and 5′-ATATCTAACACTGCCATCCC-3′; human Jak3, 5′-CAAACAC CACTCCCTGTCCT-3′ and 5′-TGGGGGTGTTCCTGAAGTAG-3′; Tyk2, 5′-GGATGGCCAGGGGCAGTAAG-3′ and 5′-GGATCTCCTC CTCGGTCGAC-3′; prostate-specific antigen, 5′-GGTCGGCACAGCC TGTTTCA-3′ and 5′-CCACGATGGTGTCCTTGATC-3′; β-actin, 5′-GACTACCTCATGAAGATCCT-3′ and 5′-GCGGATGTCCACGTCA CACT-3′. The resulting cDNA was subjected to PCR.

Immunoblot analyses. The cells were washed twice with cold PBS, and lyzed on ice in 2× sample buffer (125 mmol/L Tris, pH 6.8, 4% SDS, 10% 2-β mercaptoethanol, 20% glycerol, 0.06% bromophenol blue). The cell lysates were boiled for 3 min and resolved by 10% SDS–PAGE. Proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA), and immunoblotting was performed using rabbit anti-human Tyk2 antibody (Santa Cruz Biotechnology; 1:1000) or rabbit anti-human β-tubulin antibody (Santa Cruz Biotechnology) as an internal loading control. Goat anti-rabbit antibody conjugated by HRP (Bio-Rad, 1:3000 dilution) were used as a secondary antibody. Immunoreactive proteins were visualized with ECL detection reagents (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry. Serial 4-mm-thick sections were deparaffinized in three changes of xylene and rehydrated through a graded series of ethanol decreasing from 100% to 70%. The sections were immersed in citrate buffer (pH. 6.0) and autoclaved at 120 °C for 5 min and then placed in 3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity. Nonspecific protein binding was blocked by incubating the section for 30 min–1 h in 5% goat serum. Next, the sections were incubated overnight at 4 °C in polyclonal rabbit Tyk2 antibody (Santa Cruz Biotechnology). Sections were then processed for immunohistochemistry using the EnVisionTM+ system (DAKO, Denmark). We examined 70 samples from prostate cancer patients.

Matrigel invasion assay. Membrane inserts (8-μm pore size) for 24-well transwell plates were prepared by coating with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. DU145 cells were placed in the upper chamber at a density of 2×10^4 cells/insert. Medium containing 5 nM uPA (R&D Systems, Minneapolis, MN) was added to the lower chamber as a chemoattractant. To inhibit the invasion, 200 nM of Tyk2 siRNA/200 nM oligofectamine or 100 μM of tyrphostin A1 was added to the medium. After 24 h, the upper surface of the inserts was wiped with cotton swabs, and the inserts were stained with Trypan blue. Cells that migrated through the Matrigel and the filter pores to the lower surface were counted in five random high-power fields per insert using a light microscope.

Results

Expression analyses of Tyk2 in prostate cancer cell lines and tissues

We first analyzed the expression of Jaks in MCF-7, LNCaP, PC-3, TSU-Pr1 and DU145 cells by RT-PCR (Fig. 1A). Amplified products for four members of the Jak family, Tyk2, Jak1, Jak2, and Jak3, were clearly detected in these cell lines. We also found that prostate-specific antigen was expressed by LNCaP cells which is androgen sensitive prostate cancer cell line as previously described [3]. Although we tested one androgen sensitive prostate cancer cell line LNCaP, there was no difference of the expression level of Jaks mRNAs between androgen sensitive cells and insensitive cells. We next examined the expression of Tyk2 in human prostate cancer tissue specimens by immunohistochemistry with rabbit anti-human Tyk2 antiserum. The reactivity for Tyk2, shown as brown color, was higher in cancerous than in noncancerous glands in the same field (Fig. 1B). The staining for Tyk2 was heterogeneous and predominately located in the cytoplasm of prostate cancer cells. Of 70 samples from prostate cancer patients, Tyk2 staining was clearly detected in 17 (24.3%). Further studies are needed to accurately determine the correlation between Tyk2 expression in the cancerous tissues and clinical and pathological variables.

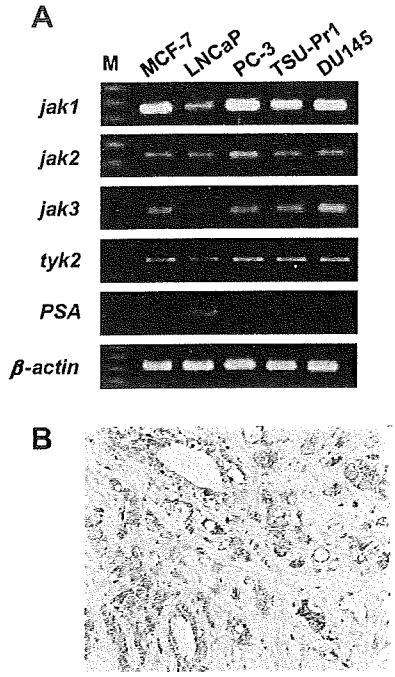

Fig. 1. (A) RT-PCR analysis of Jak family kinases mRNA levels in prostate cancer cell lines. MCF-7 is a breast cancer-derived cell line. (B) Immunohistochemical analysis of Tyk2 expression in prostate cancer tissues. Strong staining is observed in cancerous cells. Histopathological evaluation was done by a pathologist (Y.T.) and hematoxylin and eosin staining shows malignant features of these cells (data not shown).

Interference of Tyk 2 signaling dose not inhibit proliferation of prostate cancer cells

We next examined the role of Tyk2 in cell proliferation using a 96-well-based MTS assay. The experiments were carried out with cells at several different passages and with different batches of transfected cells. At a concentration of 100 μM, tyrphostin A1, a tyrosine kinase inhibitor, did not inhibit the growth rate of DU145 cells (Fig. 2A). Down-regulation of Tyk2 expression using a siRNA also did not inhibit the growth rate of DU145 cells, even though the expression of Tyk2 was decreased (Fig. 2B). These results suggest that signaling by Tyk2 does not affect the proliferation of DU145 cells. Finally, uPA did not affect the growth rate of DU145 cells (Fig. 2C).

Tyk2 signaling involved in invasiveness of prostate cancer cells through uPA

Recent reports suggest that Tyk2 regulates cell migration by mediating uPA activation of phosphatidylinositol 3-kinase [10]. Although uPA is known to regulate the invasiveness of prostate cancer cells, the underlying molecular mechanisms are still unclear. To determine whether Tyk2 participates in the promotion of invasiveness by uPA, we performed Matrigel invasion chamber assays. DU-145 cells were placed in transwells containing Matrigel-coated porous

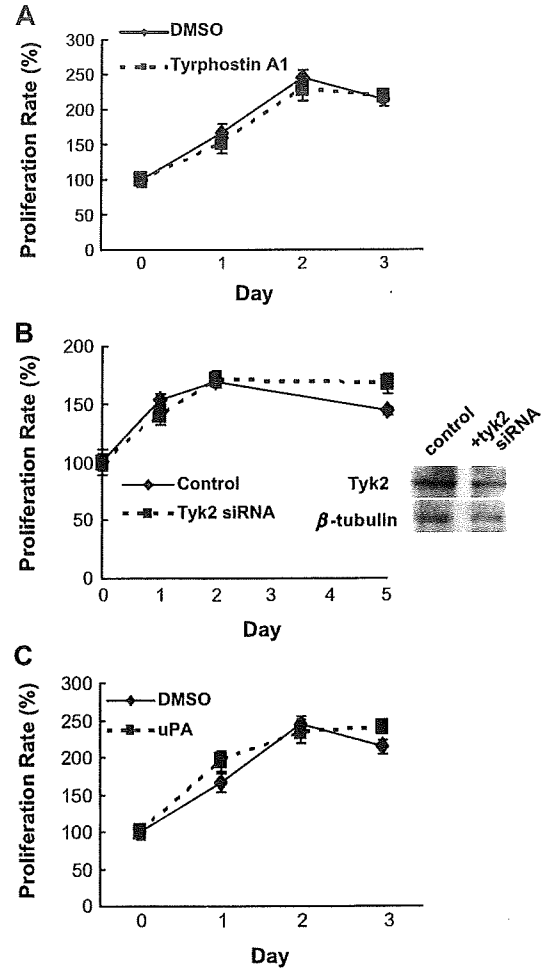

Fig. 2. Role of Tyk2 signaling in the growth of DU145 cells. (A) Tyrphostin A1 did not affect the growth of DU145 cells. (B) The siRNA targeting Tyk2 also did not affect the growth of DU145 cells. (C) uPA did not change a proliferation rate of DU-145 cells. The number of live cells was determined by MTS assay. These assays were repeated two times with different batches of transfected cells, and each cell sample was done in triplicate.

membranes, and the lower chamber was filled with medium with or without uPA. After 24 h, cells that migrated through the Matrigel basement membrane matrix and the filter pores to the lower surface of the membrane were counted by light microscopy. Treatment of cells with tyrphostin A1 inhibited the invasiveness in the presence or absence of uPA in the lower chamber of the transwell (Fig. 3A). In addition, cells transfected with the Tyk2 siRNA, showed decreased invasiveness compared to cells transfected with control siRNA (Fig. 3B). The results with the Tyk2 inhibitor and using the siRNA indicated that inhibition of Tyk2 signaling reduces the invasiveness of prostate cancer cells *in vitro*.

Discussion

In prostate cells, transformation, tumorigenesis, and metastasis can be caused by alterations in cellular regulation,

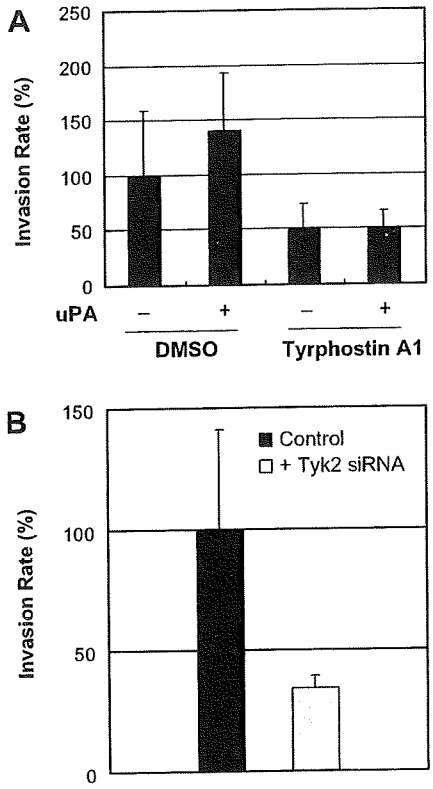

Fig. 3. The enhancement of invasion by uPA is suppressed by blockade of Tyk2 signaling in DU-145 cells. (A) The enhancement of invasiveness by uPA was suppressed by tyrphostin A1, an inhibitor of Tyk2 phosphorylation, in DU145 cells. (B) Down-regulation of Tyk2 expression with a siRNA inhibited the invasiveness of uPA-treated DU145 cells. Assays examining the effect of siRNA were repeated three times with different batches of transfected cells.

especially by dysregulation of signaling by PTKs, including receptors for transforming growth factorα, epidermal growth factor, insulin-like growth factor 1, fibroblast growth factors, hepatocyte growth factor, platelet-derived growth factor, nerve growth factors, and interleukin-6 [11]. For example, immunocytochemical analysis has shown that platelet-derived growth factor receptor-α is expressed in bone marrow metastases from prostate tumors [4]. Also, overexpression of HER-2/neu in primary prostate cancer and in metastatic sites of prostate tumors has been detected before and after hormone therapy [12]. In addition, in prostate cancer cells, interleukin-6 enhances cell growth and causes a parallel activation of the Stat3 signaling pathway [13]. Although some of the signaling components have been investigated in prostate cancer cells, only a few of these PTK genes have been shown to influence their invasiveness and metastatic potential.

CD44 has been implicated in a number of important biologic processes, including lymphocyte homing and activation, hematopoiesis, and tumor progression and metastasis [14]. CD44 may be a marker for immature and progenitor cells in prostate tissues. We previously identified Jak1 and Tyk2 using a PCR-based strategy to screen for PTKs in CD44-positive prostate cells [3]. To our knowledge, this is the first report that Tyk2 plays a significant role in mediating the enhancement of prostate cancer invasion by uPA. We found that prostate cancer cells express Jak family kinases, and in the current studies, we concentrated the role of Tyk2. Blockade of Tyk2 expression using a siRNA significantly suppressed the promotion of human prostate cancer cell invasiveness by uPA. In addition, the Tyk2 inhibitor tyrphostin A1 suppressed the invasiveness of human prostate cancer cells. We also showed that tissue samples from prostate cancer patients are positive for Tyk2.

The activation of Tyk2 signaling may depend on upstream events such as the activation of the uPA, interleukin, and interferon receptors. Furthermore, uPA is a multifunctional molecule that acts as both a proteolytic enzyme and a ligand that induces intracellular signaling. The uPA receptor mediates intracellular signaling *via* surface proteins such as integrins, growth factors receptors, and G-protein-coupled membrane proteins [15]. Several reports have shown that Jaks and Stats interfere with multiple signaling cascades, such as the Ras/mitogen-activated protein kinase pathway and activation of phosphatidylinositol 3-kinase and Src kinases [16]. For instance, Tyk2 is required for the activation of Stat3 by uPA in glomerular mesangial cells [17]. In human vascular smooth muscle cells, uPA stimulates migration *via* the uPA receptor signaling complex, which contains the Tyk2 and phosphatidylinositol 3-kinase [10]. Together with these previous findings, our results suggest that Tyk2 is one of the key molecules in mediating uPA receptor signaling in prostate cancer cells.

Our results also suggest that Tyk2 signaling may contribute to the metastasis of prostate cancer. Further studies are needed to determine whether inhibition of Tyk2 attenuates the metastasis of prostate cancer cells in *in vivo*. We are currently examining the correlation between the expression of Tyk2 in prostate cancer and the pathological and clinical variables. Several inhibitors of Jak protein kinase have been developed as molecular-targeted chemotherapeutic agents [18]. Jak kinase generally functions as a modulator at the intersection of multiple signal transduction pathways. Thus, targeting Tyk2 with specific drugs may be useful for therapeutic intervention in prostate cancer.

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