Table 2. Changes in trends in patient and treatment characteristics for Japan and the United States

Parameter	Japan			U.S.		
	1996–1998[a]	1999–2001[b]	Trends	1994[c]	1999[d]	Trends
Patient characteristics						
T stage >3	64%	46%	↓	9%	7%	→
PSA ≥20 ng/ml	55%	50%	→	12%	19%	→
GS ≥8	31%	35%	→	19%	15%	→
Treatment characteristics						
High dose (≥ 72 Gy)	2%	8%	→	3%	45%	↑
CT-based RT planning	81%	86%	→	71%	96%	↑
Hormone therapy usage	86%	90%	→	8%	51%	↑

GS, Glascow Score; RT, radiotherapy
[a] Ogawa et al.[10]
[b] Ogawa et al.[11]
[c] Zietman et al.[24]
[d] Zelefsky et al.[15]

Table 3. Radiation dose and hormone therapy usage distribution in Japan and the United States

Parameter	Japan (%)		United States (%)
	1996–1998[a]	1999–2001[b]	1999[c]
Radiation dose (Gy)			
<68	76.3	47.5	16.0
68 to <72	22.5	45.0	39.0
72 to <76	1.3	7.5	32.0
76–80	0	0	13.0
Hormone therapy usage			
Favorable	76.5	72.0	31.0
Intermediate	85.4	91.8	54.0
Unfavorable	87.1	91.1	79.0

[a] Data reanalyzed from the 1996–1998 Japan PCS results
[b] Ogawa et al.[13]
[c] Zelefsky et al.[15]

disease than their U.S. counterparts during approximately the period of 1990s.

Comparison of patterns of treatment among Japan, Germany and the United States

A previous comparison study by Ogawa et al. identified considerable differences in the patterns of care for prostate cancer between Japan and the United States[12] (Table 1). The current study also identified many differences in the patterns of radiotherapy not only between Japan and the United States but also between Japan and Germany. With regard to equipment, conformal radiotherapy was administered to only 43% of the patients in Japan versus 80% of patients in the United States and 100% in Germany. With regard to radiation doses, the mean total radiation dose for Germany was 69.1 Gy versus 66.0 Gy in Japan. Radiation doses employed in the United States were significantly higher than those used in Japan (Table 3), with almost half (45%) of the U.S. patients receiving prescribed dose levels of ≥72 Gy. The administration of higher radiation doses in Germany and the United States probably reflects the penetration into clinical practice of various reports published during the 1990s indicating that higher radiation doses were associated with a statistically significant improvement in outcome.[16,17] On the other hand, only a small number of patients in Japan (7.5%) received the higher doses (≥72 Gy) during 1999–2001. One reason for this difference may be the lower incidence of conformal radiotherapy in Japan. As mentioned above, conformal radiotherapy was administered to 85% and 100% of patients in the United States and Germany, respectively, but to only 43% of patients in Japan. Previous PCS results indicated that treatment processes in Japanese institutions were closely related to structural immaturity in terms of equipment.[4,9,11] Therefore, to provide high-quality radiotherapy in Japan,

facilities need appropriate treatment planning capability. Modern radiotherapy requires computed tomography (CT)-based treatment planning and conformal radiotherapy to improve the target dose distribution while concomitantly reducing the dose to normal tissues.[18] Another reason for the radiation dose difference may be the high incidence of hormonal therapy in Japan. At present, it is possible that many Japanese radiation oncologists consider the higher dose levels (≥72 Gy) unnecessary for prostate cancer patients when combined with long-term hormonal therapy.

With regard to hormonal therapy, differing patterns of care in hormonal therapy were found among Japan, Germany, and the United States. Most of the patients in Japan (89.7%) received hormonal therapy in conjunction with radiotherapy, whereas this combined therapy was administered less frequently in Germany (70.5%) and the United States (51.3%). Regarding the frequency of hormonal therapy for the various risk groups, the administration of hormonal therapy to favorable-risk patients was different in Japan than in the United States (Table 3). Most of the patients (72.0%) in the favorable-risk group in Japan during 1999–2001 were treated with hormonal therapy, whereas only 31% of favorable-risk patients received hormonal therapy in the United States. Several studies from the United States have indicated that radical radiotherapy alone could control prostate cancer in patients with a favorable-risk status. Zietman indicated that a total dose of 70 Gy was sufficient to control the disease when the pretreatment PSA level was <10 ng/ml.[19] Hanks et al. found that prostate cancer patients with a pretreatment PSA level of <10 ng/ml did not benefit from a dose above 70 Gy.[20] Therefore, radical external beam radiotherapy without hormonal therapy has been the primary treatment for patients in the United States with favorable-risk disease. On the other hand, 72% of the patients in the favorable-risk group in Japan were treated with long-term hormonal therapy. The high rate of health insurance coverage for Japanese people may explain the frequent administration of hormonal therapy in Japan.[21] However, because hormonal therapy has been found to be unnecessary for favorable-risk patients in the United States,[19,20] radical external beam radiotherapy without hormonal therapy may also be the treatment of choice for favorable-risk patients in Japan.

Changing trends in the patterns of treatment between Japan and the United States

Ogawa et al. compared the changes in trends in the patterns of care, and these changes were found to be quite different between Japan and the United States[12] (Table 2). Concerning radiotherapy, the United States has seen a rapid increase in CT-based treatment planning and in the percentage of patients treated with a higher dose levels (≥72 Gy) compared to the 1994 PCS results, with almost half (44.5%) of patients being treated with these higher doses in 1999 compared with 3% in 1994. In contrast, the percentage of patients receiving higher dose levels in Japan has remained below 10%, not only for 1996–1998 but also for 1999–2001. These changing trends in higher prescribed radiation doses and CT-based radiotherapy planning in the United States between 1994 and 1999 demonstrate a drastic change in these parameters over that 5-year period, whereas only minor changes, except the significant decrease of patients treated with <68 Gy (Table 3), occurred in Japan between 1996–1998 and 1999–2001.

Concerning hormone therapy, the percentage of patients receiving hormonal therapy remained high in Japan for the periods 1996–1998 and 1999–2001, whereas the use of hormonal therapy in the United States showed a rapid increase from 1994 to 1999. The significantly increased use of hormonal therapy for high-risk patients in the United States reflects the penetration and growing acceptance of clinical trial results that have demonstrated the efficacy of these treatment approaches.[22] The randomized trial RTOG 8610 demonstrated an increase in disease-free survival for locally advanced prostate cancer patients treated with neoadjuvant total androgen blockade plus radiotherapy compared with radiotherapy alone.[23] PCS results in the United States indicate a rapid increase in the use of hormonal therapy from 1994 to 1999, whereas PCS results in Japan indicate that the use of hormonal therapy in patients with unfavorable-risk disease has remained high (>90%) (Table 3). Therefore, radiotherapy in conjunction with hormonal therapy appears to be an accepted approach for the unfavorable-risk group in both Japan and the United States.

Conclusions

Comparisons of Japanese, German, and U.S. PCS results revealed several differences in the patterns of care among these countries. Higher proportions of patients treated with radical external beam radiotherapy in Japan had advanced disease compared with those in Germany and the United States. A specific comparison between Japan and the United States shows that this trend has continued over the past several years. Patterns of care for prostate cancer in Japan significantly differ from those in Germany and the United States, especially with respect to radiation dose and the use of hormonal therapy.

Moreover, changes in trends in the patterns of care also show differences between Japan and the United States. These results suggest that in Germany and the United States, radiotherapy for prostate cancer has become widely applied as an established treatment, whereas its use in Japan was still immature and developing during the period when this national survey was conducted.

We now are analyzing 2003–2005 Japan PCS data. Repeat surveys and point-by-point comparisons with results from other countries, such as Germany and the United States, should demonstrate how external beam radiotherapy for prostate cancer is being developed and optimized for patients in Japan.

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Overexpression of XIAP expression in renal cell carcinoma predicts a worse prognosis

YOICHI MIZUTANI[1], HIROYUKI NAKANISHI[1], YONG NAN LI[1], HIROKI MATSUBARA[1], KOSUKE YAMAMOTO[1], NODOKA SATO[1], TAKUMI SHIRAISHI[1], TERUKAZU NAKAMURA[1], KAZUYA MIKAMI[1], KOJI OKIHARA[1], NATSUKI TAKAHA[1], OSAMU UKIMURA[1], AKIHIRO KAWAUCHI[1], NORIO NONOMURA[2], BENJAMIN BONAVIDA[3] and TSUNEHARU MIKI[1]

[1]Department of Urology, Kyoto Prefectural University of Medicine, Kyoto 602-8566; [2]Department of Urology, Faculty of Medicine, Osaka University, Osaka 565-0871, Japan; [3]Department of Microbiology, Immunology and Molecular Genetics, Jonsson Comprehensive Cancer Center, UCLA School of Medicine, University of California at Los Angeles, CA 90095, USA

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Abstract. X-linked inhibitor of apoptosis protein (XIAP) is the most potent caspase-inhibitory IAP family member and a negative regulator of various apoptotic stimuli. Thus, XIAP overexpression in cancer cells may select for tumor cell survival following various cytotoxic therapeutic modalities. The anatomical staging system in renal cell carcinoma (RCC) currently provides good prognostic information, albeit insufficient. We hypothesize that overexpression of XIAP in RCC may serve as a molecular prognostic marker in RCC and improve the staging of RCC. This study examined the protein level of XIAP in lysates from surgical specimens of 109 patients with RCC and 109 normal kidney specimens from the same patients. The level of XIAP expression was quantified by Western blot analysis using non-fixed fresh frozen tissues of RCCs and normal kidneys. Results indicated that the mean level of XIAP expression was higher in RCC compared to autologous normal kidney, and the XIAP expression level in 38/109 (35%) of RCC was more than 2-fold greater than that in normal kidney tissue. In Stage I/II RCC, the mean XIAP expression level was almost identical to that detected in normal kidney, whereas XIAP expression in Stage III/IV was 2.5-fold higher than that in Stage I/II RCC. Levels of XIAP expression also correlated with the grade of RCC. Patients with RCC with low XIAP expression had a longer postoperative disease-specific survival as compared to those with high expression in the 5-year follow-up. The suggested role of XIAP in the regulation of resistance in apoptosis was examined *in vitro* following treatment of RCC cell lines with XIAP antisense oligonucleotide and the cells were sensitized to both Fas-mediated and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. The present study demonstrates at the protein level that XIAP is overexpressed in RCC, and that high XIAP expression in RCC predicted a worse prognosis. In addition, XIAP antisense oligonucleotide sensitized RCC to Fas/TRAIL-induced apoptosis. These results suggest that XIAP expression in RCC may be used as a prognostic parameter, and that downregulation or inhibition of XIAP expression in RCC may reverse immune resistance.

Correspondence to: Dr Yoichi Mizutani, Department of Urology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
E-mail: ymizutan@koto.kpu-m.ac.jp

Abbreviations: BIR, baculoviral inhibitor of apoptosis protein repeat; DISC, death inducing signaling complex; DIABLO, direct inhibitor of apoptosis protein-binding protein with low pl; IAP, inhibitor of apoptosis protein; mAb, monoclonal antibody; MTT, microculture tetrazolium dye; RCC, renal cell carcinoma; Smac, second mitochondria-derived activator of caspase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis protein

Key words: XIAP, RCC, prognosis, apoptosis, TRAIL, Fas

Introduction

Renal cell carcinoma (RCC) accounts for approximately 2% of all cancer cases worldwide (1). Metastatic disease is often present at the time of diagnosis of RCC and its poor response to chemotherapy and radiotherapy determines its poor prognosis (2). Immunotherapy is relatively effective for RCC, however, the response rate is approximately 20% (3). Therefore, new therapeutic approaches are necessary for these patients with metastatic RCC.

The aggressive stage of cancer is characterized by the appearance of apoptosis-resistant cells as a result of various genetic mutations and overexpression of anti-apoptotic factors. Apoptosis can be achieved by a number of ligand receptor families, commonly called death receptors and also by various drugs or stress-induced stimuli. Death receptors bind to their respective ligands and form the death inducing signaling

complex (DISC) that is followed by a cascade mediating the apoptotic cellular events (4). The key executor of the apoptotic pathway is a group of cysteine proteases known as caspases that are present in the cytosol as inactive zymogens and are proteolytically activated by the appropriate apoptogenic agents (5). The initiator caspases become activated by association of the signaling complex of death receptors or with the apoptosome in the cytosol and trigger the activation of effector caspases. In type I cells, ligand binding to death receptors causes strong activation of caspase-8 which leads to processing and activation of caspase-3 and the subsequent induction of apoptosis. In type II cells, caspase-8 stimulation by DISC formation results in the cleavage of Bcl-2 family member BID. A fragment of BID is translocated into the mitochondria and induces the release of apoptogenic proteins, particularly cytocrome c, second mitochondria derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low pl (Smac/DIABLO), and a serine protease HTR2/OMI. The latter are blockers of caspase inhibitory function of members of the IAP family (6). Cytosolic cytocrome c forms an apoptosome complex with pro-caspase-9 and Apaf-1, which in turn releases active caspase 9, and results in the activation of the executioner caspase-3 (7).

The IAPs are endogenous caspase inhibitors (8). The X-linked IAP (XIAP) is considered the prototype of the IAP family and has been identified as a potent caspase inhibitor (9). The IAPs, originally described in baculovirus (10), function by binding to activated caspases and inhibiting their pro-apoptotic function. The IAPs contain baculovirus IAP repeat (BIR) domains. In addition to BIR domains, several IAPs also contain a RING domain which binds ubiquitin-conjugating enzymes that promote degradation of IAP-caspase complexes (11). Eight human IAPs have been reported, namely XIAP, cIAP1, cIAP2, Survivin, NIAP, Bruce, ML-IAP and ILP-2 (12). All IAPs except NIAP can bind and inhibit activated caspase-3 and 7. In addition, cIAP1, cIAP2 and XIAP can also inhibit the activity of caspase-9 (13).

XIAP is the best characterized member of the IAP family in terms of the caspase inhibitory mechanism. XIAP contains three BIR domains. BIR2 and flanking regions are responsible for binding and potently inhibiting caspase-3 and caspase-7, while BIR3 and flanking regions suppress caspase-9 (8,13). Thus, XIAP has the potential to inhibit active caspases, slow down the process at this step and inhibit apoptosis (14). These findings implied that overexpression of XIAP in tumor cells may render the cells resistant to apoptotic stimuli and may survive following therapy. XIAP is expressed in normal tissues, however, overexpression of XIAP has been demonstrated in several cancers including lung cancer and prostate cancer (15,16). In addition, down-regulation of XIAP sensitizes the resistant cancer cells to death receptor- or cytotoxic drug-induced apoptosis (17,18). These findings suggest that XIAP plays an important role in the regulation of apoptotic responses in cancer cells to both immune- and drug-mediated therapies. The present study was designed to investigate the level of XIAP expression in RCCs compared with its expression in autologous normal kidneys and determine its prognostic significance.

Patients and methods

Patients. Surgical specimens were obtained from 109 patients with RCC. These patients were selected randomly for this study. They included 81 male and 28 female patients, ranging in age from 19 to 83 years. Histologic diagnosis revealed that 99, 9 and 1 patients had clear cell carcinoma, papillary RCC and Bellini duct carcinoma, respectively. Their histologic classification and staging according to TNM classification (UICC, 6th edition, 2002) were: T1 (n=69), T2 (n=9), T3 (n=23), T4 (n=8); N0 (n=105), N1 (n=1), N2 (n=3); M0 (n=94), M1 (n=15); Stage I (n=63), Stage II (n=5), Stage III (n=17), Stage IV (n=24), and G1 (n=13), G2 (n=66), G3 (n=30), respectively. Normal kidney specimens were collected from the same 109 patients with RCC. The paired samples were histologically confirmed to be RCC and normal kidney. The specimens were stored frozen at -80˚C until use.

This study was performed after approval by a local Human Investigations Committee. Informed consent was obtained from each patient.

RCC cell lines. NC65, ACHN and Caki-1 human RCC cell lines (19,20) were maintained in monolayers on plastic dishes in RPMI-1640 medium (Gibco, Bio-cult, Glasgow, Scotland, UK) supplemented with 25 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 1% non-essential amino acid (Gibco), 100 U/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco) and 10% heat-inactivated fetal bovine serum (Gibco), hereafter referred to as complete medium.

Measurement of the level of XIAP expression in RCC and normal kidney using Western blot and quantification image analysis. The presence of XIAP protein was determined in cell lysates by Western blot analysis as previously described (21). Briefly, 20 μg of the sample proteins was electrophoresed on 7.5% polyacrylamide gels in Tris-glycin buffer and transferred onto nitrocellulose membranes. The membrane was blocked for 30 min in blocking buffer (5% skim milk in 1% Tween-PBS) and probed with first antibody (anti-XIAP monoclonal antibody (mAb): R&D systems, Minneapolis, MN) for 1 h. After being washed, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG and developed with the use of an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). The relative expression of XIAP protein was determined with a chemiluminescence imaging system and quantified by image analysis (Gel Doc 2000: Bio-Rad, Osaka, Japan).

The NC65 cell line constitutively expressed XIAP and was used as internal standard to compare assays. All samples were analyzed at the same time. Repeated measurements yielded identical results. When the level of XIAP expression in RCC samples was >2-fold higher than that found in the autologous normal kidney, it was regarded as high expression. In contrast, when the level of XIAP expression was <2-fold, it was regarded as low expression.

Reagents. Phosphorothioate oligonucleotides (XIAP antisense oligonucleotide: 5'-CTG TTA AAA GTC ATC TTC TC-3', scrambled oligonucleotide: 5'-CTT GAT AGA ATC TAC TCT CT-3') were synthesized by ß-cyanoethyl-phospho-

roramidite chemistry using an automated DNA synthesizer (Applied Biosystems, Foster city, CA) (22). Deprotection and purification of phosphorothioate oligonucleotides were carried out according to the protocol described in the user manual. Phosphorothioate oligonucleotides were checked for purity by reverse-phase high-performance liquid chromatography.

Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and anti-Fas mAb (CH-11) were purchased from Peprotech, Rocky Hill, NJ and MBL Co. Ltd., Nagoya, Japan, respectively.

Cytotoxicity assay. Microculture tetrazolium dye (MTT) assay was used to determine tumor cell lysis as previously described (23,24). Briefly, 100 μl of target cell suspension ($2x10^4$ cells) was added to each well of 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, NY), and each plate was incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, the supernatant was aspirated and tumor cells were washed three times with RPMI medium, and 200 μl of drug solution or complete medium for control was distributed in the 96-well plates. Each plate was incubated for 72 h at 37°C. Following incubation, 20 μl of MTT working solution (5 mg/ml, Sigma Chemical Co., St. Louis, MO) was added to each culture well and the cultures were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was removed from the wells and replaced with 100 μl of isopropanol (Sigma Chemical Co.) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Immunoreader, Japan Intermed Co. Ltd., Tokyo, Japan) at 540 nm. Percent cytotoxicity was calculated as follows: Percentage cytotoxicity = [1 - (absorbance of experimental wells/absorbance of control wells)] x 100.

Statistical analysis. All determinations were made in triplicate. For statistical analysis, Student's t-test was used. Postoperative disease-specific survival was determined by the Kaplan-Meier method. The Cox-Mantel test was used to establish the statistical difference in survival between RCC patients with high and low XIAP expression. In addition, multivariate Cox proportional hazards risk analysis was also used. A $p \leq 0.05$ was considered significant.

Results

Expression levels of XIAP protein in lysates from RCC cell lines, RCCs and normal kidneys. The levels of XIAP protein expression in total cell lysates derived from RCC cell lines, RCC and normal kidney specimens were determined by Western blot analysis as described in Patients and methods. The NC65, ACHN, and Caki-1 RCC cell lines all expressed XIAP, although at different levels (Fig. 1). By comparison, NC65 expressed the highest level of XIAP, while ACHN expressed the lowest level.

The levels of XIAP expression were determined in lysates derived from 109 normal kidneys and 109 RCCs. Representative data of XIAP expression in RCCs and normal kidneys from the same patients are shown in Fig. 2. Clearly, XIAP expression was observed in all normal kidney specimens.

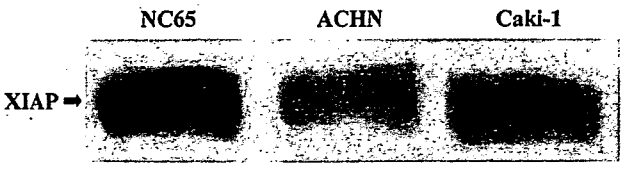

Figure 1. Expression of XIAP in RCC cell lines. XIAP expression in RCC cell lines (NC65, ACHN, Caki-1) was examined by Western blot analysis as described in Patients and methods.

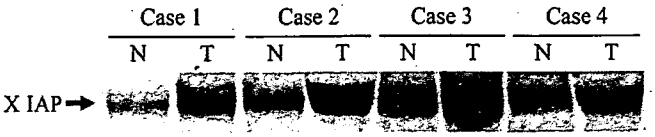

Figure 2. Expression of XIAP in RCCs and normal kidneys. XIAP expression in RCCs and normal kidneys was examined by Western blot analysis as described in Patients and methods. Cases 1-4 are representative cases. XIAP expression in RCCs was higher than that in normal kidneys. N, normal kidney, T, RCC.

Table I. The level of XIAP expression as a function of stage and grade in RCC compared to normal kidney.

RCC (stage/grade)	Ratio of XIAP expression level compared to normal kidney[a] (mean ± SE)
RCC (n=109)	1.61±0.09
Tumor stage	
I/II (n=70)	1.13±0.06
III/IV (n=39)	2.54±0.15[b]
Tumor grade	
1 (n=13)	1.14±0.15
2 (n=66)	1.33±0.08
3 (n=30)	2.51±0.21[c]

[a]Ratio of the level of XIAP expression in RCCs over normal kidneys was examined by Western blot analysis as described in Patients and methods. [b]$p<0.05$ vs. Stage I/II RCC. [c]$p<0.05$ vs. Grade 1, Grade 2 RCC.

Noteworthy, the XIAP expression in normal kidneys in patients with RCC was similar to that in patients with renal pelvic cancer or ureteral cancer (data not shown). By comparison, the level of XIAP expression in the majority of RCCs was higher than that in normal kidneys, as represented in Fig. 2. The ratios of XIAP expression in RCC over normal kidney were determined and the findings are summarized in Table I. The mean ratio of XIAP expression in RCC over normal kidney was 1.6. The mean ratio of XIAP expression in Stage I/II and Stage III/IV RCC compared to normal kidney was 1.1 and 2.5, respectively. The findings observed in the various stages were corroborated with Grades of RCC. Hence, the ratios of XIAP expression in Grade 1, Grade 2 and Grade 3

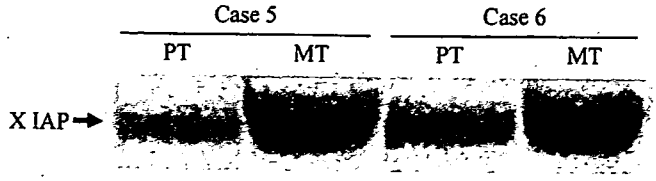

Figure 3. Expression of XIAP in primary and metastatic RCCs. XIAP expression in primary and metastatic RCCs was examined by Western blot analysis as described in Patients and methods. Cases 5 and 6 are representative cases. XIAP expression in metastatic RCCs was significantly higher than that in primary RCCs in these cases. Case 5, bone metastasis; Case 6, bone metastasis. PT, primary RCC; MT, metastatic RCC.

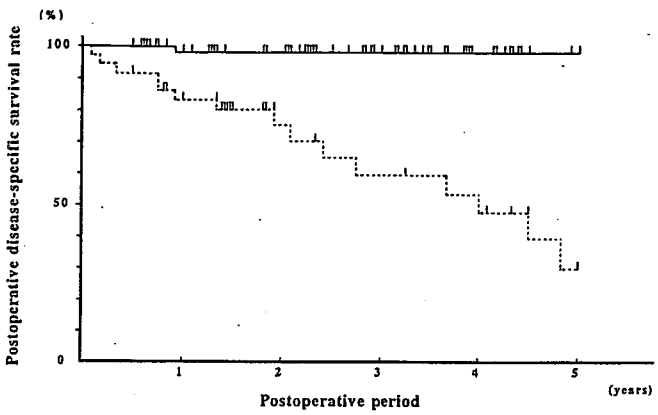

Figure 4. Relationship between XIAP expression and postoperative disease-specific survival in patients with RCC. Postoperative disease-specific survival of RCC patients undergoing radical nephrectomy was determined by the Kaplan-Meier method. Patients with RCC were divided into two groups, namely, those with low XIAP expression and those with high expression. Patients with RCC with low XIAP expression had a longer disease-specific survival as compared to those with high expression in the 5-year follow-up ($P<0.01$ by Cox-Mantel test). Solid lines, 71 patients with low XIAP expression; interrupted lines, 38 patients with high XIAP expression.

RCCs compared to normal kidney were 1.1, 1.3 and 2.5, respectively.

Preliminary experiments in two patients with metastatic RCC demonstrated that XIAP expression was significantly higher in metastatic RCC compared to that found in primary RCC (Fig. 3). The level of XIAP expression in clear cell RCC was similar to that in papillary RCC (data not shown).

Altogether, these data demonstrate that there was a significant increase of the level of XIAP expression in RCCs as compared to the level found in normal kidneys. Further, the levels of XIAP expression detected correlated with the stage of tumor progression and the increase of the histologic grade of RCC.

Correlation between the level of XIAP expression and postoperative disease-specific survival in patients with RCC. RCC patients undergoing radical nephrectomy were evaluated for the postoperative clinical course. The postoperative disease-specific survival was estimated by Kaplan-Meier analysis. Based on the analysis, patients with RCC were divided into two groups, namely, those with high XIAP expression (>2-fold increase of normal levels of XIAP expression) and those with low expression (<2-fold) as described in Patients and methods. Patients with RCC with low levels of XIAP expression had a longer disease-specific survival as compared to those with high expression in the 5-year follow-up (Fig. 4). Noteworthy, only one patient with RCC with low XIAP expression died during the course of this study, and the expression of XIAP was very high in metastatic tumor (representative case 6, Fig. 3). Multivariate analysis of the data showed that the level of XIAP expression was an independent prognostic factor in patients with RCC ($p=0.0368$). These findings suggest that the level of XIAP expression in RCC may be a prognostic indicator, and that, more specifically, low XIAP expression in RCC may be a good prognostic sign.

Table II. Enhancement of Fas-mediated cytotoxicity against NC65 cells by XIAP antisense oligonucleotide.

Concentration of XIAP antisense oligonucleotide (μg/ml)	% Cytotoxicity (mean ± SD)[a] Concentration of anti-Fas mAb (ng/ml)			
	0	10	100	1000
0	0	52.6±6.2	63.6±3.8	75.1±9.3
0.1	0.6±0.3	62.7±6.2[b]	71.2±7.4	78.0±8.7
1	1.5±0.3	68.4±7.2[b]	75.0±6.0[b]	82.1±7.2
10	2.9±0.2	70.3±6.4[b]	85.5±3.3[b]	92.7±1.8[b]

[a]The cytotoxic effect of anti-Fas mAb and XIAP antisense oligonucleotide used in combination on NC65 cells was assessed in a 3-day MTT assay. The results are expressed as the mean ± SD of 3 different experiments. [b]Values in the combination treatment are significantly higher than those achieved by treatment with anti-Fas mAb alone plus those with XIAP antisense oligonucleotide alone at $p<0.05$.

Table III. Enhancement of TRAIL-mediated cytotoxicity against NC65 cells by XIAP antisense oligonucleotide.

Concentration of XIAP antisense oligonucleotide (μg/ml)	% Cytotoxicity (mean ± SD)[a] Concentration of TRAIL (ng/ml)			
	0	1	10	100
0	0	0.9±1.1	3.4±1.2	12.8±2.4
0.1	0.9±0.3	8.7±0.8	12.2±2.0	27.0±4.2[b]
1	1.5±1.2	15.2±1.6[b]	17.9±2.1[b]	31.0±3.2[b]
10	2.5±2.5	19.9±2.0[b]	22.8±3.2[b]	34.6±3.2[b]

[a]The cytotoxic effect of TRAIL and XIAP antisense oligonucleotide used in combination on NC65 cells was assessed in a 3-day MTT assay. The results are expressed as the mean ± SD of 3 different experiments. [b]Values in the combination treatment are significantly higher than those achieved by treatment with TRAIL alone plus those with XIAP antisense oligonucleotide alone at $p<0.05$.

XIAP regulates tumor cell sensitivity to Fas-mediated and TRAIL-mediated cytotoxicity. Since XIAP expression was upregulated in RCC, we then examined the effect of XIAP antisense oligonucleotide treatment of RCC cell lines upon tumor growth as well as Fas/TRAIL-induced cytotoxicity. XIAP antisense oligonucleotide had no direct effect on the growth of the NC65 RCC cell line (Tables II and III). However, treatment of NC65 cells with XIAP antisense oligonucleotide enhanced Fas-mediated cytotoxicity (Table II). When NC65 cells were treated with a combination of XIAP antisense oligonucleotide and TRAIL, significant potentiation of cytotoxicity and synergy were obtained (Table III). Treatment with XIAP antisense oligonucleotide also sensitized NC65 cells to TNF-α-mediated cytotoxicity (data not shown). In addition, when the ACHN cell line was used as a target, treatment with XIAP antisense oligonucleotide also potentiated Fas/TRAIL-induced cytotoxicity (data not shown).

These findings suggest that high expression of XIAP in RCC may be associated with immune-resistance, and that downregulation of XIAP expression may enhance Fas/TRAIL-mediated apoptosis in RCC.

Discussion

Tumor cells develop several mechanisms to evade cell death by apoptosis. This anti-apoptotic mechanism provides the tumor cells an advantage to escape both host-immune destruction as well as develop resistance to chemotherapeutic and radiation-induced cell death. Therefore, tumor cells that manage to resist apoptotic stimuli can progress and metastasize. Several mechanisms have been reported that underlie tumor cell escape from apoptosis-induced signaling and particularly perturbations in the main apoptotic signaling pathways, namely the death receptor type I and mitochondria type II pathways. Thus, gene products that regulate the apoptotic pathways have been shown to be de-regulated in various cancers and responsible, in large part, for resistance. Among the gene products that regulate apoptosis, the IAP family has been shown to play a major role in several cancers (4). For example, survivin has been shown to be overexpressed in many tumors (25,26) and has been also shown to be a prognostic marker (27,28). The expression and prognostic significance of XIAP, however, has been rarely studied.

Evidence is presented that XIAP expression was up-regulated in RCC, compared with normal kidney, and that the level of XIAP expression positively correlated with both the progression of the stage and the increase of the grade of RCC. Furthermore, this study shows that RCC patients with low XIAP expression had a longer disease-specific survival as compared to those with high expression in the 5-year follow-up. Although the data reported here corresponds to a small number of patients during a short-term follow-up, these findings suggest that XIAP in RCC may play an important role in regulating the malignant potential and apoptosis and may be of prognostic value in RCC.

Our findings here are in agreement with those reported recently by Ramp *et al* (29). These investigators used immunohistochemistry for their analysis, whereas we have examined total protein levels derived from fresh tumor lysates and normal kidneys by Western blot analysis. It is conceivable that detection of XIAP protein by immunohistochemistry may not be measuring the whole XIAP protein but only degraded XIAP products that are recognized by the antibody. The Western blot analysis quantified the XIAP expression levels based on expected molecular weight. In addition, Ramp *et al* primarily examined only clear cell RCC, whereas in our studies we have also analyzed papillary RCC which showed similar patterns to clear cell RCC. Comparable results to RCC were reported for the prognostic significance of XIAP in acute myeloid leukemia (30). Conflicting results have been reported for small cell lung carcinoma (15,31).

The present study has shown that the level of XIAP expression in RCC predicted the clinical outcome. The precise reasons responsible for this relationship remain unclear at present. Since XIAP is an anti-apoptotic molecule, it is reasonable to assume that in spite of treatments, clones of cells which overexpress XIAP can grow more easily and rapidly than those which express XIAP at a low level. In addition, this study has shown that XIAP was overexpressed in metastatic RCC compared to primary RCC. Although more cases need to be examined to confirm it, these findings suggest that XIAP antagonists may provide a therapeutic means of preventing metastasis and growth of RCC.

Immunotherapy including interleukin-2 and interferon-α is relatively effective against metastatic RCC, and the overall response rate of immunotherapy and/or chemotherapy has gradually improved (3,32,33). However, the response rate is approximately 20%, and metastasis and recurrence of RCC still remain major problems in the therapy for RCC (3). Therefore, new therapeutic approaches are required for the patients. Enhanced XIAP expression in RCC compared to normal kidney identifies XIAP as a molecular therapeutic target. Our observation that downregulation of XIAP expression in RCC by antisense oligonucleotide resulted in high sensitivity to Fas/TRAIL-mediated killing may be of potential clinical importance in the management of patients with RCC. The endogenous level of XIAP expression in RCC may be too high to induce apoptosis. Thus, immunotherapy in combination with XIAP antagonists may be a promising strategy against RCC (17,18).

The role of XIAP in the regulation of tumor cell resistance to anticancer chemotherapeutic drugs and immunotherapy has been reported. The XIAP level determines tumor cell sensitivity to various drugs (34) and radiation (9). Also, XIAP levels regulate tumor cell sensitivity to Fas ligand- and TRAIL-induced apoptosis (35,36). The underlying mechanisms responsible for regulating overexpression of XIAP are not clear. Tumor cells have been shown to have high levels of constitutively activated NF-κB and NF-κB has been shown to be implicated in the transcriptional regulation of XIAP. XIAP is also regulated post-transcriptionally (9). XIAP contains a specific ring finger domain which has been shown to promote protein ubiquitination and auto-degradation in a proteasome-dependent manner (9). Smac/DIABLO accelerates XIAP auto-ubiquitination and self degradation and modulates protein expression levels. Noteworthy, we have recently reported in RCC that the expression of Smac/DIABLO is significantly decreased and is an independent prognostic factor for RCC (21). Thus, it is possible that the down-regulation of Smac/DIABLO contributed, in large part, to the stabilization and

high level of XIAP expression in RCC. Current studies are examining whether this correlation is found in the same patients.

Smac/DIABLO was recently identified as a protein that is released from mitochondria in response to apoptotic stimuli (37,38). Smac/DIABLO is able to bind to IAP family members, and XIAP is a predominant Smac/DIABLO binding protein. Smac/DIABLO binds to XIAP, displaces XIAP from caspase-9, promotes cleavage of effector caspases, and induces apoptosis (6,39). In addition, our previous study demonstrated that Smac/DIABLO expression was downregulated in RCC, and that no Smac/DIABLO expression in RCC predicted a worse prognosis (21). Therefore, the measurement of Smac/DIABLO expression as well as XIAP expression may be necessary for an accurate prediction of prognosis in RCC patients and an accurate evaluation of the efficacy of the therapy using XIAP antagonists in combination with immunotherapy.

Cancer therapy using TRAIL or anti-DR4/5 mAb is currently being investigated in clinical trials due to low toxicity to normal tissues (40,41). However, not all cancers respond to TRAIL, and resistance to apoptosis induced by TRAIL has been demonstrated to be overcome by anticancer agents (42,43), XIAP antisense oligonucleotide or small molecule antagonists of XIAP (17,18,22). Thus, analysis of the expression of XIAP in RCC may be helpful for determining therapeutic modalities such as TRAIL therapy and immunotherapy.

In conclusion, this study demonstrated that XIAP expression was upregulated in RCC, and that high XIAP expression was a poor prognostic sign. Furthermore, decreased XIAP expression by antisense oligonucleotide rendered resistant RCC cells sensitive to Fas/TRAIL-mediated cytotoxicity. These findings suggest that the assessment of XIAP expression may be useful in the management of RCC. Since XIAP expression could be used as a prognostic parameter in patients with RCC, an accurate prediction of prognosis may help select patients for more intensive surgical or immunotherapeutic approaches in combination with XIAP antagonists.

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Molecular Features of Hormone-Refractory Prostate Cancer Cells by Genome-Wide Gene Expression Profiles

Kenji Tamura,[1,2] Mutsuo Furihata,[3] Tatsuhiko Tsunoda,[4] Shingo Ashida,[2] Ryo Takata,[5] Wataru Obara,[5] Hiroki Yoshioka,[1] Yataro Daigo,[1] Yasutomo Nasu,[6] Hiromi Kumon,[6] Hiroyuki Konaka,[7] Mikio Namiki,[7] Keiichi Tozawa,[8] Kenjiro Kohri,[8] Nozomu Tanji,[9] Masayoshi Yokoyama,[9] Toru Shimazui,[10] Hideyuki Akaza,[10] Yoichi Mizutani,[11] Tsuneharu Miki,[11] Tomoaki Fujioka,[5] Taro Shuin,[2] Yusuke Nakamura,[1] and Hidewaki Nakagawa[1]

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; Departments of [2]Urology and [3]Pathology, Kochi University, Kochi Medical School, Nankoku, Japan; [4]Laboratory for Medical Informatics, SNP Research Center, RIKEN (Institute of Physical and Chemical Research), Yokohama, Japan; [5]Department of Urology, Iwate Medical University, Morioka, Japan; [6]Department of Urology, Okayama University, Okayama, Japan; [7]Department of Urology, Kanazawa University, Kanazawa, Japan; [8]Department of Urology, Nagoya City University, Nagoya, Japan; [9]Department of Urology, Ehime University, Shitsukawa, Japan; [10]Department of Urology, Tsukuba University, Tsukuba, Japan; and [11]Department of Urology, Kyoto Prefectural Medical School, Kyoto, Japan

Abstract

One of the most critical issues in prostate cancer clinic is emerging hormone-refractory prostate cancers (HRPCs) and their management. Prostate cancer is usually androgen dependent and responds well to androgen ablation therapy. However, at a certain stage, they eventually acquire androgen-independent and more aggressive phenotype and show poor response to any anticancer therapies. To characterize the molecular features of clinical HRPCs, we analyzed gene expression profiles of 25 clinical HRPCs and 10 hormone-sensitive prostate cancers (HSPCs) by genome-wide cDNA microarrays combining with laser microbeam microdissection. An unsupervised hierarchical clustering analysis clearly distinguished expression patterns of HRPC cells from those of HSPC cells. In addition, primary and metastatic HRPCs from three patients were closely clustered regardless of metastatic organs. A supervised analysis and permutation test identified 36 up-regulated genes and 70 down-regulated genes in HRPCs compared with HSPCs (average fold difference > 1.5; $P < 0.0001$). We observed overexpression of *AR, ANLN*, and *SNRPE* and down-regulation of *NR4A1, CYP27A1*, and *HLA-A* antigen in HRPC progression. *AR* overexpression is likely to play a central role of hormone-refractory phenotype, and other genes we identified were considered to be related to more aggressive phenotype of clinical HRPCs, and in fact, knockdown of these overexpressing genes by small interfering RNA resulted in drastic attenuation of prostate cancer cell viability. Our microarray analysis of HRPC cells should provide useful information to understand the molecular mechanism of HRPC progression and to identify molecular targets for development of HRPC treatment. [Cancer Res 2007;67(11):5117–25]

Introduction

Prostate cancer is the most common malignancy in males and the second leading cause of cancer-related death in the United States and Europe (1). The incidence of prostate cancer has been increasing significantly in most of developed countries due to prevalence of Western-style diet and explosion of the aging population (1, 2). The screening using serum prostate-specific antigen (PSA) lead to dramatic improvement of early detection of prostate cancer and resulted in an increase of the proportion of patients with a localized disease that could be curable by surgical and radiation therapies (1, 2). However, 20% to 30% of these prostate cancer patients still suffer from the relapse of the disease (3–5).

Androgen/androgen receptor (AR) signaling pathway plays a central role in prostate cancer development, and the prostate cancer growth is usually androgen-dependent at a relatively early stage (3–5). Hence, most of the patients with relapsed or advanced disease respond well to androgen ablation therapy, which suppresses testicular androgen production by surgical castration or by administration of an agonist(s) to luteinizing hormone-releasing hormone (LH-RH) and antiandrogen drugs. Nonetheless, they eventually acquire androgen-independent and more aggressive phenotype that has been termed hormone-refractory prostate cancers (HRPCs). Recently, the combination of docetaxel and prednisone was established as the new standard of care for HRPC patients (6, 7), but they are not curable and their survival benefit on HRPC patients is very limited. Hence, many groups are now attempting various approaches to identify novel molecule targets or signaling pathways that contribute to growth of HRPC (8).

Several studies using *in vitro* prostate cancer cell lines and mouse models have shown that the progression to HRPC could be associated with increased levels of *AR* expression, implicating that *AR* down-regulation by means of small interfering RNA (siRNA) or other methods should suppress tumor growth even in HRPCs (8–11). The *AR* gene was overexpressed in most of HRPCs, in 10% to 20% of which amplification of the *AR* gene was observed (12). In addition, a subset (<10%) of HRPCs was found to have somatic mutations in the *AR* gene, which could enhance ligand response (13). As consequence, expressions of several AR-regulated genes were reactivated even under androgen depletion (3, 14–16). Furthermore, the AR pathway in HRPCs was consider to rely on alterations in growth factors, such as insulin-like growth factor

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Accession codes: The complete microarray data set is available from the Gene Expression Omnibus (GSE6811).

Requests for reprints: Hidewaki Nakagawa, Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5375; Fax: 81-3-5449-5124; E-mail: hidewaki@ims.u-tokyo.ac.jp.

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(17), HER-2 (18), and cytokines, such as interleukin-6 (19), which could modify the AR activity. Overexpression of these growth factors or coactivators in HRPCs might change cancer cells to be independent of the AR signaling (8, 14, 20). Despite these latest advances in molecular analysis of AR pathways, the mechanisms by which prostate cancer cells survive and acquire their more aggressive phenotype after androgen ablation therapy are still not well understood.

In this report, to characterize the molecular feature of clinical HRPCs, we did genome-wide cDNA microarray analysis of cancer cells purified from HRPC tissues by means of laser microbeam microdissection (LMM) and identified several deregulated genes in HRPCs, some of which might be involved in their androgen-independence and aggressive phenotype. These data should shed a light on a better understanding of the molecular mechanisms underlying clinical HRPCs and could suggest candidate genes whose products could serve as molecular targets for development of novel treatment for HRPC.

Materials and Methods

Patients and tissue samples. Tissue samples were obtained with informed consent from 43 HRPC patients undergoing prostatic needle biopsy, bone biopsy, transurethral resection of the prostate (TUR-P), and "warm" autopsy. Clinical HRPC was defined by elevation of serum PSA

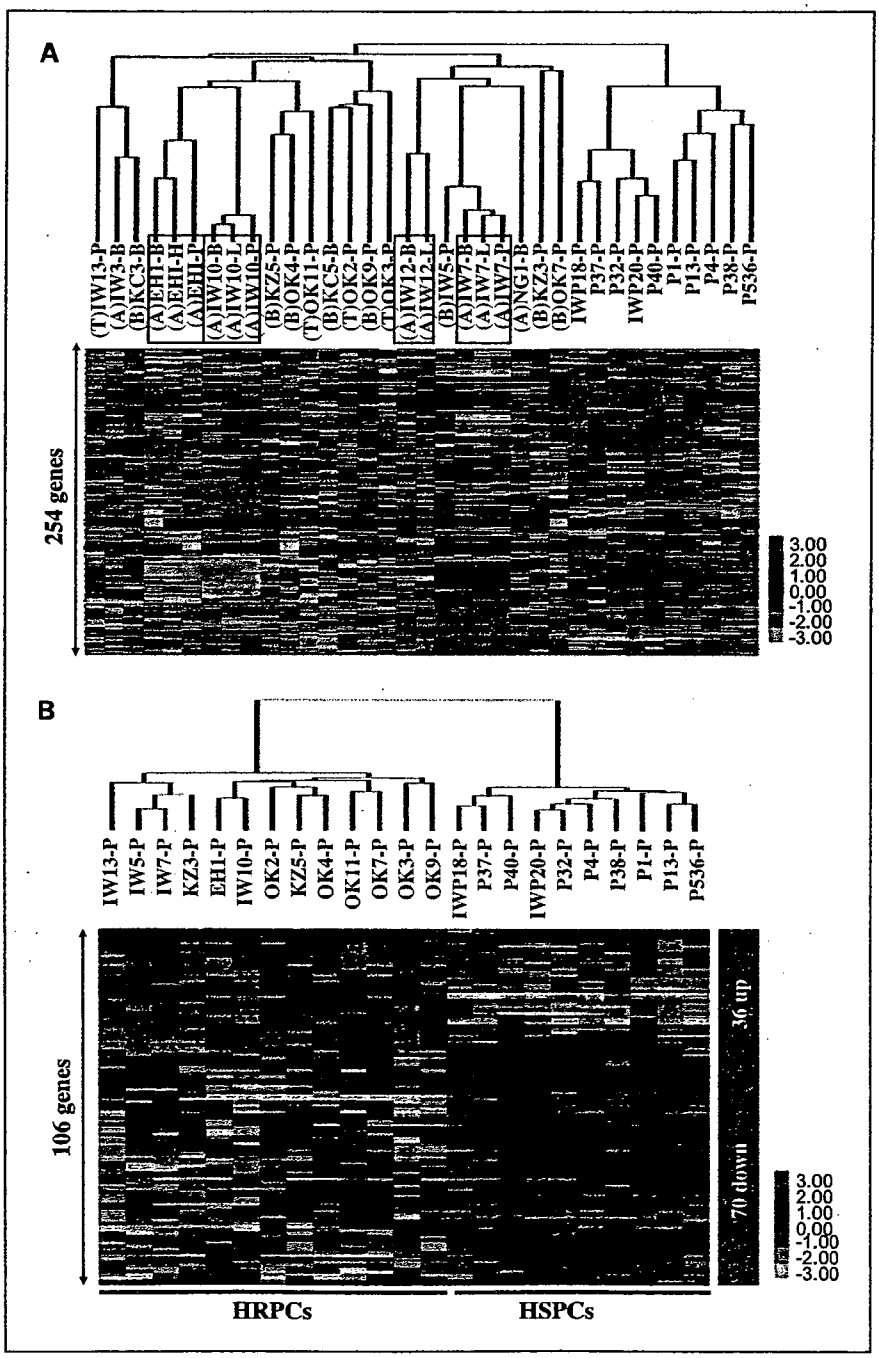

Figure 1. *A*, dendrogram of an unsupervised hierarchical clustering analysis of 254 genes (*vertical columns*) across 35 prostate cancers (*horizontal rows*). The unsupervised hierarchical clustering analysis clearly distinguished 25 HRPCs (*red*) from 10 HSPCs (*blue*). Small subsets of cluster constituted by metastatic HRPC cells (*-B*, bone metastasis; *-L*, lymph node metastasis; *-H*, liver metastasis) and HRPC cells at the primary site (*-P*, prostate) from the same individuals (*black boxes*). Tissue procurement methods for each HRPC specimen (*A*, autopsy; *B*, biopsy; *T*, TUR-P). All HSPC tissues were procured by radical prostatectomy. *B*, dendrogram of a supervised analysis of 106 genes (*vertical columns*) across 13 HRPCs at the prostate and 10 HSPCs (*horizontal rows*). Each cell in the matrix represents the expression level of a single transcript in a single sample. *Red* and *green*, transcript levels, above and below the median for that gene across all samples. *Black*, unchanged expression; *gray*, no detectable expression. The 36 up-regulated genes and 70 down-regulated genes that can distinguish HRPC cells from HSPC cells are listed in Tables 1 and 2, respectively.

Table 1. Up-regulated genes in the progression to HRPC

Accession no.	Difference	*P* value	Symbol	Gene name
NM_003094.2	3.306386323	3.77×10^{-7}	*SNRPE*	*Small nuclear ribonucleoprotein polypeptide E*
NM_018685.2	3.219579761	3.32×10^{-6}	*ANLN*	*Anillin, actin binding protein (scraps homologue, Drosophila)*
AA976712.1	3.182541782	5.99×10^{-7}	*TMEM46*	*Transmembrane protein 46*
AI357641.1	3.146687689	7.51×10^{-6}	*CDKN2C*	*Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)*
W67209.1	3.087139806	5.93×10^{-8}	*SESN3*	*Sestrin 3*
DB340904.1	2.985071675	8.00×10^{-8}	*AR*	*AR, 3′ untranslated region*
NM_018144.2	2.890854838	3.62×10^{-9}	*SEC61A2*	*Sec61 α2 subunit (S. cerevisiae)*
NM_016448.1	2.811368667	7.78×10^{-5}	*DTL*	*Denticleless homologue (Drosophila)*
Z74616.1	2.538137903	1.92×10^{-5}	*COL1A2*	*Collagen, type I, α2*
NM_000090.2	2.491419709	5.12×10^{-5}	*COL3A1*	*Collagen, type III, α1*
R41754.1	2.447849423	1.19×10^{-10}	*TMEM132B*	*Transmembrane protein 132B*
NM_006607.1	2.440825064	5.09×10^{-7}	*PTTG2*	*Pituitary tumor-transforming 2*
U73727.1	2.333606143	2.40×10^{-5}	*PTPRU*	*Protein tyrosine phosphatase, receptor type, U*
AK021786.1	2.319361143	3.24×10^{-7}	*C17orf72*	*Chromosome 17 open reading frame 72*
AA910060.1	2.236962817	8.99×10^{-5}		*EST*
AA621719.1	2.220224258	8.14×10^{-6}	*SMC4*	*SMC4 structural maintenance of chromosomes 4-like 1*
AK024438.1	2.198639235	7.77×10^{-5}	*ZFP41*	*Zinc finger protein 41 homologue (mouse)*
NM_006229.1	2.192827851	6.66×10^{-5}	*PNLIPRP1*	*Pancreatic lipase-related protein 1*
AA195210.1	2.170830793	2.46×10^{-6}	*DKFZP761M1511*	*Hypothetical protein DKFZP761M1511*
AK096164.1	2.04647546	4.34×10^{-6}	*EIF2C2*	*Eukaryotic translation initiation factor 2C, 2*
AF035594.1	2.002516001	6.59×10^{-6}	*PRKCA*	*Protein kinase C, α*
NM_004442.5	1.921607125	1.06×10^{-5}	*EPHB2*	*EPH receptor B2*
AK096873.1	1.78604725	7.02×10^{-5}	*NPEPL1*	*Aminopeptidase-like 1*
NM_005733.1	1.739425422	2.56×10^{-5}	*KIF20A*	*Kinesin family member 20A*
AA757026.1	1.678056856	8.32×10^{-5}		*EST*
X63679.1	1.644063667	9.85×10^{-7}	*TRAM1*	*Translocation associated membrane protein 1*
NM_001211.4	1.636586307	9.84×10^{-5}	*BUB1B*	*BUB1 budding uninhibited by benzimidazoles 1 homologue β*
NM_017915.2	1.61745888	3.12×10^{-5}	*C12orf48*	*Chromosome 12 open reading frame 48*
NM_014176.1	1.589009131	1.95×10^{-8}	*UBE2T*	*Ubiquitin-conjugating enzyme E2T (putative)*
NM_006265.1	1.56936713	1.03×10^{-7}	*RAD21*	*RAD21 homologue (S. pombe)*
NM_007220.3	1.563745272	3.02×10^{-13}	*CA5B*	*Carbonic anhydrase VB, mitochondrial*
BC044310.1	1.563713145	2.78×10^{-5}	*TNK2*	*Tyrosine kinase, non-receptor, 2*
N51406.1	1.541763744	3.79×10^{-5}	*RP11-393H10.2*	*Hypothetical protein FLJ14503*
NM_005055.3	1.525830663	5.79×10^{-5}	*RAPSN*	*Receptor-associated protein of the synapse, 43 kDa*
NM_016275.3	1.519432356	1.63×10^{-5}	*SELT*	*Selenoprotein T*
NM_006988.3	1.504404832	6.73×10^{-5}	*ADAMTS1*	*ADAM metallopeptidase with thrombospondin type 1 motif, 1*

NOTE: Difference: the average fold difference in expression level between HRPCs and HSPCs.

levels at three consecutive times and/or enlargement of tumor in spite of androgen ablation therapy. All of the samples were embedded in OTC Compound (Tissue-Tek) immediately after tissue procurement and stored at −80°C until their use. Histopathologic diagnoses were made by a single pathologist (M.F.) before LMM, and H&E-stained sections from adjacent frozen tissues were prepared to confirm the histologic diagnosis. Among the 43 HRPC patients we obtained, 25 primary or metastatic tumor specimens from 18 HRPC patients had sufficient amounts and good quality of RNAs for our microarray analysis. Tissue procurement method and therapeutic treatments for these 18 HRPC patients are shown in Supplementary Table S1. We microdissected cancer cells from these frozen slides by means of LMM (EZ cut system with a pulsed UV narrow beam-focus laser, SL Microtest GmbH). Simultaneously, 10 hormone-sensitive prostate cancers (HSPCs) or hormone-naive prostate cancers were also microdissected from 10 untreated operable cases undergoing radical prostatectomy, and normal prostatic (NP) epithelial cells were also microdissected from one benign prostatic hyperplasia patient and four bladder cancer patients, where we confirmed no apparent prostate cancers or prostatic intraepithelial neoplasias histopathologically.

Genome-wide cDNA microarray analysis and acquisition of data. LMM and T7-based RNA amplification were done as described previously (21). Amplified RNAs of 2.5 μg each were labeled by reverse transcription with Cy5-dCTP for cancer cells or Cy3-dCTP for normal cells (Amersham Biosciences) as described previously (21). We fabricated a genome-wide cDNA microarray with 36,864 cDNAs selected from the UniGene database (build no. 131) of the National Center for Biotechnology Information. Construction, hybridization, washing, and scanning were carried out according to methods described previously (21). Signal intensities of Cy3 and Cy5 from the 36,864 spots were quantified and analyzed by substituting backgrounds with ArrayVision software (Imaging Research, Inc.). Subsequently, the fluorescent intensities of Cy5 (cancer) and Cy3 (normal control) for each target spot were adjusted so that the mean Cy3/Cy5 ratio of 52 housekeeping genes was equal to one. Because data with low-signal intensities are less reliable, we determined a cutoff value on each slide, and we excluded genes from further analysis when both the Cy3 and the Cy5 dyes yielded signal intensities lower than that of the cutoff value. For other genes, we calculated the Cy5/Cy3 ratio using the raw data of each sample.

Hierarchical clustering and statistical analysis for genome-wide gene expression profiles. We applied an unsupervised hierarchical

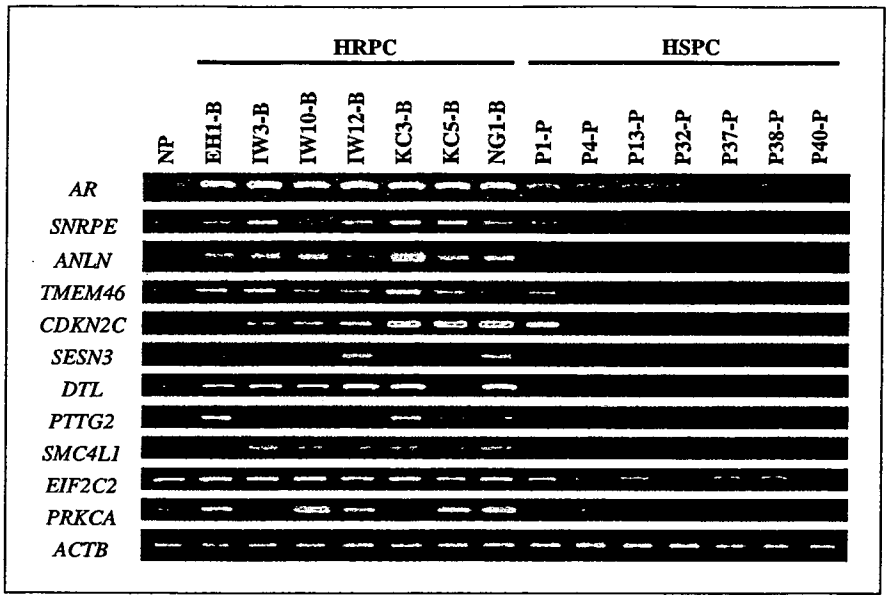

Figure 2. Semiquantitative RT-PCR confirmed the elevated expression of 11 genes that could distinguish HRPC cells from HSPC cells (7 HRPCs and 7 HRPCs microdissected from prostrate cancer tissues). *ACTB* was used to quantify the each of cDNA contents.

clustering method to both genes and tumors, excluding genes, for which both Cy3 and Cy5 fluorescence intensities were below the cutoff value. To obtain reproducible clusters for classification of the 35 tumors, we selected 254 genes (about one percentile of the whole human transcripts) for which valid data were obtained in 80% of the experiments and whose expression ratios varied by SDs of >1.75. We log transformed the fluorescence ratio for each spot and then median centered the data for each sample to remove experimental biases. The unsupervised clustering analysis was done with web-available software (Cluster version 3.0 and TreeView version 1.0.12) written by Eisen.[12] As a supervised analysis, we applied a random permutation test to identify genes that were expressed at a significantly different level between the two groups (HRPC: 1 versus HSPC: 2). For each gene (g), we used a measure of correlation $P(g, c) = [\mu_1(g) - \mu_2(g)] / [SD_1(g) + SD_2(g)]$, which reflects the difference of average (μ) between the groups relative to the SD within the groups (22). The results are compared with the corresponding distribution obtained for random idealized expression patterns c^*, obtained by randomly permuting the coordinates of $c = (c_1, c_2, \ldots, c_n)$, where $c_i = +1$ or 0 according to whether the i-th sample belongs to group 1 or group 2 (22). The random permutations were applied so that each group had a constant number of samples. Using thus obtained empirical distribution of null hypothesis, we calculated P value for each gene. We selected 106 genes for which valid data were obtained by P value of <0.0001, average fold difference of >1.5, and one group present of >60%.

Semiquantitative reverse transcription-PCR and real-time quantitative reverse transcription-PCR. Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's instruction, treated with DNase I (Roche Diagnostics), and reversely transcribed to single-stranded cDNA using random hexamer or oligo(dT)$_{12-18}$ primer with SuperScript reverse transcriptase II (Invitrogen). We prepared appropriate dilutions of each single-strand cDNA followed by normalizing cDNA content using β-*actin* (*ACTB*) as a quantitative control, showing PCR using single-strand cDNA as PCR templates. The primers of each transcripts were the following: *ACTB* [5′-TTGGCTTGACTCAGGATTTA-3′ (forward) and 5′-ATGCTATCACCTCCCCTGTG-3′ (reverse)], *SNRPE* [5′-CAAGTGAATATGCGGATAGAAGG-3′ (forward) and 5′-CCATCTTGTAGTAACACGAGGGT-3′ (reverse)], *ANLN* [5′-GCTGCGTAGCTTACAGACTTAGC-3′ (forward) and 5′-AAGGCGTTTAAAGGTGATAGGTG-3′ (reverse)], and *AR* [5′-GTGCTGTCCTTGGAATTAATCTG-3′ (forward) and 5′-AACAGAACACTAGCGCTTGGAG-3′ (reverse)]. The PCR primers of other transcripts will be informed when they are requested. The conditions for PCR are follows: initial denaturation at 95°C for 5 min; 23 cycles (for *ACTB*, *SNRPE*, and *AR*), 30 cycles (for *TMEM46*, *CDKN2C*, *DTL*, *PTTG2*, *SMC4*, *EIF2C2*, and *PRKCA*), or 35 cycles (for *SESN3*) of denaturation at 95°C for 30 s; annealing at 55°C for 30 s; and elongation at 72°C for 30 s on a GeneAmp PCR system 9700 (PE Applied Biosystems). We carried out real-time quantitative PCRs using a Prism 7700 sequence detector (PE Applied Biosystems) with the SYBR Premix ExTaq (TaKaRa) in accordance with the manufacturer's instructions. The primers of each transcript were the following: *AR* [5′-GAGAGAGAGAAAGAAAGCATCACAC-3′ (forward) and 5′-AACACTAGCGCTTGGAGCTG-3′ (reverse)], *PSA* [5′-CCAGACACTCACAGCAAGGA-3′ (forward) and 5′-ATCCCATGCCAAAGGAAGAC-3′ (reverse)], and *NKX3.1* [5′-TGGTTTGTGAATCCATCTTGC-3′ (forward) and 5′-AACAGGCTGTCTGGGTGAAA-3′ (reverse)]. *ACTB* was used to normalize each expression and the primer sequences of *ACTB* were described above.

Immunohistochemistry. Paraffin-embedded tissue sections were deparaffinized, subjected to treatment with microwave at 360 W for 1 min four times in antigen retrieval solution, high pH (DAKO), and then treated with peroxidase blocking reagent (DAKO) followed by protein block reagent (DAKO). Immunohistochemical study was carried out using the Ventana automated IHC systems (DiscoveryTM, Ventana Medical systems, Inc.). Sections were incubated with a 1:100 diluted solution of a mouse monoclonal antibody (mAb; NCL-AR-318, Novocastra) against the NH_2-terminal portion of the human AR overnight at 4°C. The automated protocol is based on an indirect biotin-avidin system using a biotinylated universal secondary antibody and diaminobenzidine substrate with hematoxylin counterstaining.

siRNA-expressing constructs and colony formation/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. We used siRNA expression vector (psiU6BX) for RNA interference effect to the target genes as described previously (23). Plasmids designed to express siRNA were prepared by cloning of double-stranded oligonucleotides into psiU6BX vector. The oligonucleotide sequences of target sequences for *SNRPE* and *ANLN* are as follows: sense strand sequence for SNRPE-si1 (5′-GGAAAGAATGAAGTGCCTT-3′), SNRPE-si2 (5′-GCTGGTAGGCAAATTGTTA-3′), SNRPE-si3 (5′-GGTGAATGCAGAAGTGTAT-3′), siANLN (5′-CCAGTTGAGTCGACATCTG-3′), and siEGFP (5′-GAAGCAGCACGACTTCTTC-3′) as a negative control. Prostate cancer cell line 22Rv1 was purchased from American Type Culture Collection, and 2×10^6 22Rv1 cells were grown on 10-cm dishes, transfected with psiU6-SNRPE (si1-3) or psiU6-ANLN or psiU6-EGFP using Fugene 6 reagent (Roche) according to the manufacturer's instruction, and cultured in appropriate medium containing 800 μg/mL geneticin (Sigma-Aldrich) for 2 weeks. The cells were fixed with 100% methanol and

[12] http://rana.lbl.gov/EisenSoftware.htm

Table 2. Down-regulated genes in the progression to HRPC

Accession no.	Difference	*P* value	Symbol	Gene name
BC092418.1	5.558869662	1.22×10^{-6}	*MYBPC1*	*Myosin binding protein C, slow type*
X04325.1	5.062445046	1.06×10^{-5}	*GJB1*	*Gap junction protein, β 1, 32 kDa*
NM_001150.1	4.826495535	7.40×10^{-10}	*ANPEP*	*Alanyl (membrane) aminopeptidase*
NM_024080.3	4.090779772	9.33×10^{-12}	*TRPM8*	*Transient receptor potential cation channel, subfamily M member 8*
NM_002443.2	3.771232027	4.03×10^{-9}	*MSMB*	*Microseminoprotein, β*
L13740.1	3.204461772	7.41×10^{-35}	*NR4A1*	*Nuclear receptor subfamily 4, group A, member 1*
NM_000784.2	3.176792485	2.03×10^{-12}	*CYP27A1*	*Cytochrome P450, family 27, subfamily A, polypeptide 1*
AA243967.1	3.119889956	3.91×10^{-5}	*ACPP*	*Acid phosphatase, prostate*
NM_138342.2	3.049236542	5.45×10^{-5}	*LOC89944*	*Hypothetical protein BC008326*
AF266280.1	2.994099827	7.11×10^{-6}	*LGALS3*	*Lectin, galactoside-binding, soluble, 3 (galectin 3)*
AB010419.1	2.95051338	2.21×10^{-5}	*CBFA2T3*	*Core-binding factor, runt domain, α subunit 2; translocated 3*
NM_003407.1	2.906880911	1.24×10^{-9}	*ZFP36*	*Zinc finger protein 36, C3H type, homologue (mouse)*
NM_032801.3	2.863996452	1.93×10^{-8}	*JAM3*	*Junctional adhesion molecule 3*
NM_004417.2	2.840006269	2.20×10^{-10}	*DUSP1*	*Dual specificity phosphatase 1*
NM_005252.2	2.779578672	2.58×10^{-6}	*FOS*	*V-fos FBJ murine osteosarcoma viral oncogene homologue*
Y11339.2	2.673481738	1.55×10^{-13}	*ST6GALNAC1*	*ST6-N-acetylgalactosaminide α-2,6-sialyltransferase 1*
L19871.1	2.519745453	1.64×10^{-9}	*ATF3*	*Activating transcription factor 3*
BC016952.1	2.420887227	4.47×10^{-10}	*CYR61*	*Cysteine-rich, angiogenic inducer, 61*
N70019.1	2.393780488	1.28×10^{-5}	*MT1M*	*Metallothionein 1M*
NM_005139.2	2.30234995	7.04×10^{-20}	*ANXA3*	*Annexin A3*
NM_005767.3	2.292587029	2.07×10^{-5}	*P2RY5*	*Purinergic receptor P2Y, G-protein coupled, 5*
M62829.1	2.268933663	5.38×10^{-5}	*EGR1*	*Early growth response 1*
R38989.1	2.188002228	8.97×10^{-8}	*SH3BGRL2*	*SH3 domain binding glutamic acid-rich protein like 2*
NM_001669.2	2.186821444	5.88×10^{-11}	*ARSD*	*Arylsulfatase D*
NM_001584.1	2.17939094	3.05×10^{-6}	*MPPED2*	*Metallophosphoesterase domain containing 2*
L02950.1	2.16892321	1.08×10^{-9}	*CRYM*	*Crystallin, mu*
NM_032592.1	2.148649429	3.29×10^{-5}	*PHACS*	*1-Aminocyclopropane-1-carboxylate synthase*
NM_015267.1	2.123686657	6.64×10^{-7}	*CUTL2*	*Cut-like 2 (Drosophila)*
R42862.1	2.085435644	2.09×10^{-6}		*EST*
NM_014861.1	2.024649382	7.07×10^{-9}	*KIAA0703*	*KIAA0703 gene product*
AL832642.2	2.022123591	5.38×10^{-6}	*CD44*	*CD44 molecule (Indian blood group)*
NM_005891.1	2.020215882	8.92×10^{-11}	*ACAT2*	*Acetyl-CoA acetyltransferase 2*
AF070632.1	2.020131066	2.51×10^{-15}		*EST*
AA742701.1	2.017839777	4.39×10^{-10}	*LCP1*	*Lymphocyte cytosolic protein 1 (L-plastin)*
NM_014841.1	2.007560931	5.59×10^{-5}	*SNAP91*	*Synaptosomal-associated protein, 91 kDa homologue (mouse)*
M62831.1	2.005374607	3.91×10^{-11}	*IER2*	*Immediate early response 2*
NM_178835.2	1.975475229	3.50×10^{-5}	*LOC152485*	*Hypothetical protein LOC152485*
NM_024709.2	1.96745649	3.60×10^{-5}	*C1orf115*	*Chromosome 1 open reading frame 115*
DA313595.1	1.961835426	2.05×10^{-5}	*HLA-A*	*MHC, class I, A*
X04481.1	1.915026305	6.40×10^{-6}	*C2*	*Complement component 2*
NM_173653.1	1.912986031	2.69×10^{-9}	*SLC9A9*	*Solute carrier family 9 (sodium/hydrogen exchanger), member 9*
NM_002228.3	1.872688976	3.02×10^{-5}	*JUN*	*V-jun sarcoma virus 17 oncogene homologue (avian)*
X07549.1	1.852416069	1.51×10^{-7}	*CTSH*	*Cathepsin H*
L05779.1	1.835437702	7.24×10^{-8}	*EPHX2*	*Epoxide hydrolase 2, cytoplasmic*
BC012037.1	1.832746195	5.02×10^{-6}	*NBL1*	*Neuroblastoma, suppression of tumorigenicity 1*
L13288.1	1.830209324	1.63×10^{-8}	*VIPR1*	*Vasoactive intestinal peptide receptor 1*
NM_004842.2	1.817118309	7.01×10^{-12}	*AKAP7*	*A kinase (PRKA) anchor protein 7*
NM_016573.2	1.813774769	1.80×10^{-11}	*GMIP*	*GEM interacting protein*
AF237813.1	1.811745708	1.59×10^{-8}	*ABAT*	*4-Aminobutyrate aminotransferase*
U47025.1	1.752036006	3.21×10^{-8}	*PYGB*	*Phosphorylase, glycogen; brain*
NM_005080.2	1.745150156	1.13×10^{-8}	*XBP1*	*X-box binding protein 1*
BQ182018.1	1.709983737	1.27×10^{-7}	*SSU72*	*SSU72 RNA polymerase II CTD phosphatase homologue*
AK129574.1	1.702855987	1.32×10^{-5}	*DOCK5*	*Dedicator of cytokinesis 5*
AK026400.1	1.691754511	1.85×10^{-7}	*FLJ42562*	*Similar to echinoderm microtubule associated protein like 5*
X51345.1	1.688349706	1.63×10^{-9}	*JUNB*	*Jun B proto-oncogene*
NM_014010.3	1.66324122	6.53×10^{-5}	*ASTN2*	*Astrotactin 2*
NM_000527.2	1.647252497	1.35×10^{-5}	*LDLR*	*Low density lipoprotein receptor (familial hypercholesterolemia)*
NM_014174.2	1.645153682	1.13×10^{-5}	*THYN1*	*Thymocyte nuclear protein 1*
AA523303.1	1.63829997	2.70×10^{-8}	*DEF6*	*Differentially expressed in FDCP 6 homologue (mouse)*

(Continued on the following page)

Table 2. Down-regulated genes in the progression to HRPC (Cont'd)

Accession no.	Difference	*P* value	Symbol	Gene name
X12548.1	1.620216872	1.61×10^{-8}	*ACP2*	*Acid phosphatase 2, lysosomal*
BX648582.1	1.611126501	5.56×10^{-9}	*SPRY2*	*Sprouty homologue 2 (Drosophila)*
NM_005951.1	1.598742883	5.62×10^{-8}	*MT1H*	*Metallothionein 1H*
NM_014553.1	1.594509479	8.36×10^{-5}	*TFCP2L1*	*Transcription factor CP2-like 1*
M96824.1	1.593941987	1.88×10^{-9}	*NUCB1*	*Nucleobindin 1*
U79240.1	1.567475917	5.60×10^{-8}	*PASK*	*PAS domain containing serine/threonine kinase*
NM_006633.1	1.564791961	7.99×10^{-5}	*IQGAP2*	*IQ motif containing GTPase activating protein 2*
NM_017679.2	1.547638171	5.30×10^{-9}	*BCAS3*	*Breast carcinoma amplified sequence 3*
NM_000295.3	1.545151445	6.49×10^{-6}	*SERPINA1*	*Serpin peptidase inhibitor, clade A, member 1*
AL390079.1	1.52908521	4.32×10^{-8}	*LOC58489*	*Hypothetical protein from EUROIMAGE 588495*
NM_000282.2	1.527984534	3.77×10^{-10}	*PCCA*	*Propionyl CoA carboxylase, α polypeptide*

NOTE: Difference: the average fold difference in expression level between HRPCs and HSPCs.

stained with 0.1% of crystal violet-H_20 for colony formation assay. In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell viability was measured using Cell Counting Kit-8 (Dojindo) at 10 days after the transfection. Absorbance was measured at 490 nm, and at 630 nm as reference, with a Microplate Reader 550 (Bio-Rad). Preliminarily, knockdown effects of these siRNA expression vectors on the endogenous expression of the target genes were validated 7 days after the transfection by reverse transcription-PCR (RT-PCR) using the primers described above.

Results

Sample collecting and hierarchical clustering analysis of expression profiles. For this study, we collected 77 frozen specimens from 43 HRPC patients through prostatic needle biopsy, bone biopsy, TUR-P, or "warm" autopsy. Nearly two thirds of specimens were not qualified for LMM and microarray analysis through the pathologic evaluation by H&E staining or after evaluation of their RNA quality, and finally, RNAs of 25 HRPC specimens from 18 HRPC patients were available for further microarray analysis. All of these 18 patients had been treated with maximum androgen blockade with LH-RH agonist treatment or surgical castration as shown in Supplementary Table S1. Tissue procurements from six autopsies were done immediately after patient death. Twenty-five HRPC specimens from 18 patients included 13 HRPCs at the primary site (prostate), 8 bone metastases, 3 lymph node metastases, and 1 liver metastasis. Simultaneously, HSPC cells were also microdissected from 10 untreated operable patients undergoing radical prostatectomy, and NP epithelial cells were also microdissected from five non–prostate cancer patients. These NP cells from five males were used as a normal mixture control for our cDNA microarray analysis. We successfully microdissected HRPC cells, HSPC cells, and NP cells from each clinical sample to exclude the contamination of stromal cells and host organ cells at the metastatic sites, such as bone marrow cells, lymphocytes, and hepatocytes (Supplementary Fig.).

An unsupervised hierarchical clustering analysis using expression patterns of 254 genes that we selected based on strict conditions (i.e., valid data obtained in 80% of the experiments and expression ratios that varied by >1.75 SDs) clearly classified the 35 tumors into two major groups, the HRPC and HSPC groups (Fig. 1*A*). This unsupervised hierarchical clustering analysis also classified multiple tumors from the same individuals to small subgroups regardless to the metastatic organs (Fig. 1*A*, *black boxes*), suggesting little influence on expression patterns by host organs of their metastatic sites due to the precise microdissection technique in our laboratory.

Identification of deregulated genes in the progression from HSPC to HRPC. To extract genes that showed significantly differential expression levels in HRPCs and HSPCs, we carried out a random permutation test using the expression profiles of 13 HRPCs at the prostate and 10 HSPCs. We selected 13 HRPCs at the primary site (prostate) among 25 HRPC specimens for this random permutation test because multiple HRPC samples from one individual showed quite similar patterns in the unsupervised hierarchical clustering analysis (Fig. 1*A*) and also because potential microenvironmental influence from host organs should be excluded as much as possible. The supervised analysis and this random permutation test (average fold difference > 1.5; $P < 0.0001$) identified 36 up-regulated genes and 70 down-regulated genes in HRPCs compared with HSPCs, which were considered to be involved in the presumably HRPC progression, that is to say, their androgen-independent growth and more aggressive or malignant phenotype (Fig. 1*B*). Table 1 listed 36 up-regulated genes in HRPC, including *AR, small nuclear ribonucleoprotein peptide E* (*SNRPE*), and *anillin, actin binding protein* (*ANLN*). Notably, the expression level of *AR* in HRPC cells was much higher than that in HSPCs, which was concordant with several previous reports studying the cell line models (9–11) and clinical samples (9). As shown in Fig. 2, semiquantitative RT-PCR validated overexpression of 11 genes in HRPC cells. On the other hand, Table 2 listed 70 down-regulated genes in the progression to HRPCs, including *NR4A1, CYP27A1*, and *HLA-A*.

AR expression and activity in clinical HRPC cells. We further analyzed AR protein expression in clinical HRPCs by immunohistochemistry and the transcriptional level of AR-regulated genes by real-time quantitative PCR, which should reflect the actual AR activity as a transcriptional factor in the nucleus. Immunohistochemical analysis for AR using 6 HRPCs and 16 HSPCs showed the positive staining in the nuclei of all HRPC, HSPC, and normal prostate. The staining intensity or patterns in HRPC cells (Fig. 3*A*) were similar to those in HSPC (Fig. 3*B*) and NP cells (Fig. 3*B*), although the transcript levels of *AR* in HRPC cells were much

higher that in HSPC and NP cells, which were analyzed by semiquantitative PCR (Fig. 2) and also quantified by real-time PCR (Fig. 3*D*). Furthermore, real-time quantitative PCR showed that transcriptional levels of AR-regulated or downstream genes, *PSA* and *NKX3.1* (24), in HRPC cells were also similar to those in HSPC and NP cells (Fig. 3*D*), in spite of >10 times overexpression of the *AR* transcript in HRPC cells. These findings implicated that clinical HRPC cells are likely to maintain AR activity in the cell by overexpressing *AR* mRNA, under very low level of circulating testicular androgen, but the stabilized AR protein and the actual AR activity levels as a transcriptional factor in the nuclei of HRPC cells were similar to that in HSPC cells and NP cells.

Knockdown effect of *ANLN* and *SNRPE* on HRPC cell growth. To investigate the contribution of non-AR pathways or genes to HRPC phenotype, we selected *ANLN* and *SNRPE*, whose expressions were most significantly high in HRPCs (Fig. 2; Table 1). We constructed several vectors, designed to express siRNA specifically to *ANLN* (siANLN) and *SNRPE* (si1-3), and transfected each of them into prostate cancer cell line 22Rv1, which expressed *ANLN* and *SNRPE* at high level. The transfection with siANLN showed the significant knockdown effect on the *ANLN* transcript (Fig. 4*A*) and resulted in drastic reduction of the numbers of colonies (Fig. 4*B*, *left*) as well as those of the viable cells measured by MTT assay (Fig. 4*B*, *right*), whereas the transfection of a negative control (siEGFP) did not show any effect. Among three siRNA constructs to *SNRPE*, *SNRPE*-si1 and *SNRPE*-si3 significantly knocked down *SNRPE* expression (Fig. 4*C*) and caused drastic reduction of the numbers of colonies (Fig. 4*D*, *left*) as well as those of the viable cells measured by MTT assay (Fig. 4*D*, *right*), whereas the transfection of the other plasmid (si2) or a negative control (siEGFP) showed no or little knockdown effect on *SNRPE* expression and did not affect prostate cancer cell viability. These findings suggested that non-AR pathways represented by overexpressing genes in HRPC, such as *SNRPE* and *ANLN*, could play some important roles in the prostate cancer cell viability as well as the AR pathway.

Discussion

Most of the patients with relapsed or advanced prostate cancer respond well to androgen ablation therapy. However, the tumors eventually acquire androgen-independent and more aggressive phenotype for which most anticancer drugs or therapies are not effective, finally leading prostate cancer patients to death. (8). In this study, we approached to the molecular mechanism of acquirement of this more aggressive phenotype by analyzing human HRPC clinical samples but not by the use of cell lines or mouse models. The gene expression studies for clinical HRPCs have been very limited thus far, largely due to difficulties to obtain

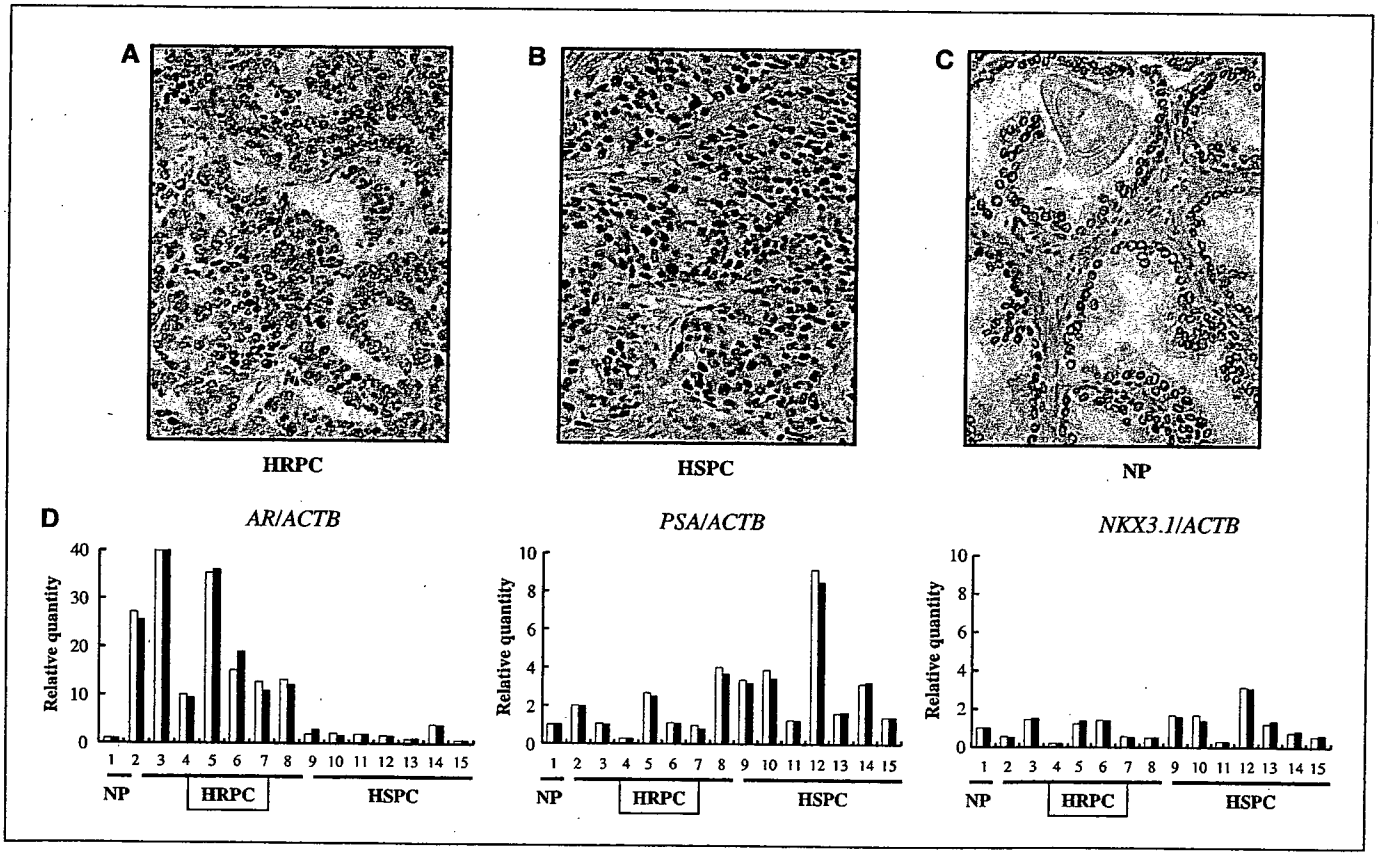

Figure 3. Immunohistochemical analysis of prostate cancer tissues by anti-AR mAb. Immunoreactivity with anti-AR antibody exhibited positive staining in the nuclei of HRPC cells (*A*), HSPC cells (*B*), and NP epithelial cells (*C*). Their staining intensity or patterns in HRPC cells is similar to those in HSPC and NP cells, although the mRNA level of *AR* in HRPC cells was much higher than in HSPC and NP cells (Figs. 2 and 3*D*). *D*, *left*, real-time quantitative PCR showed >10 times overexpression of *AR* transcript in HRPC cells (*samples 2–8*) comparing with that of HSPC cells (*samples 9–15*) and NP cells (*sample 1*). On the other hand, transcriptional levels of *PSA* (*middle*) and *NKX3.1* (*right*) of HRPC cells, which reflect AR activity, were similar to those of HSPC cells and NP cells. *ACTB* was used to quantify each of the cDNA contents, and the relative quantity (*Y-axis*) was calculated so that the expression in NP cells was one. Real-time PCR was done duplicated for each sample (*white* and *black columns*).

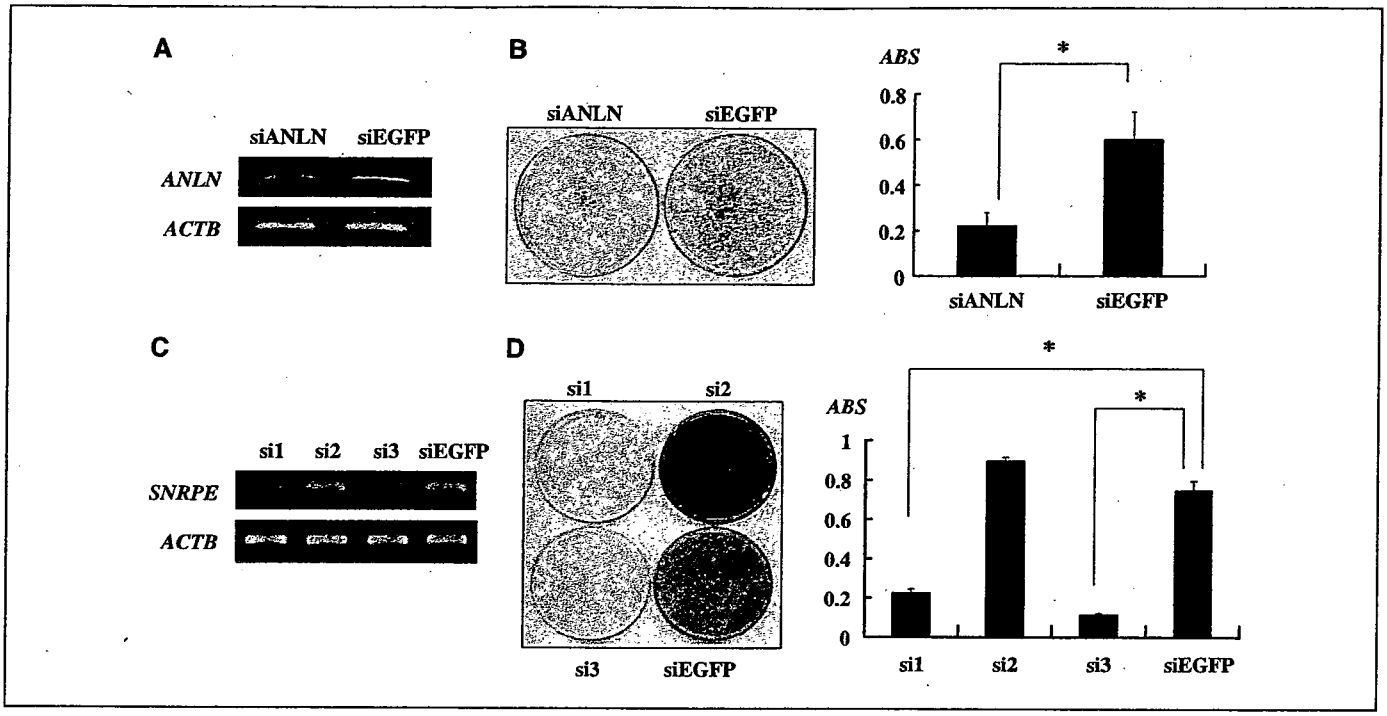

Figure 4. Knockdown of *ANLN* and *SNRPE* by siRNA in prostate cancer cells attenuated their growth and viability. *A* and *C*, knockdown effect of siRNA on *ANLN* and *SNRPE* in prostate cancer cell line 22Rv1 was evaluated by semiquantitative RT-PCR using cells transfected with each of siRNA-expressing vectors to *ANLN* (siANLN), *SNRPE* (si1-3), and a negative control vector (siEGFP). *ACTB* was used to quantify RNAs. Colony formation assay was assessed on 22Rv1 cells (*B* and *D*, *left*) transfected with each of indicated siRNA-expressing vectors to *ANLN* (siANLN), *SNRPE* (si1–si3), and a negative control vector (siEGFP). Cells were visualized with 0.1% crystal violet staining after 14-d incubation with geneticin. MTT assay was done for each of 22Rv1 (*B* and *D*, *right*) transfected with indicated siRNA-expressing vectors to *ANLN* (siANLN), *SNRPE* (si1–si3), or a negative control vector (siEGFP). *Columns*, average after 14-d incubation with geneticin; *bars*, SD. *ABS*, absorbance at 490 nm, and at 630 nm as reference, measured with a microplate reader (*Y-axis*). These experiments were carried out in triplicate. *, $P < 0.01$, Student's *t* test.

appropriate frozen HRPC samples (25, 26). Hence, we are confident that our precise genome-wide expression profiles of clinical HRPC cells are very valuable. The random permutation test comparing the expression profiles of 13 HRPCs with those of 10 HSPCs identified 36 up-regulated genes and 70 down-regulated genes in HRPCs (Tables 1 and 2). Some of such genes were considered to be associated with their androgen-independent growth and more aggressive phenotypes of clinical HRPCs. Among the 36 up-regulated genes in HRPCs, at first, we focused on *AR* overexpression. In spite of *AR* transactivation of mRNA in HRPC cells, the amount of stabilized AR protein in the nucleus and AR activity measured by the transcriptional levels of its downstream target genes (*PSA* and *NLX3.1*) in HRPC cells were similar to those in HSPC and normal prostate epithelial cells. Several reports suggested that even under low level of circulating testicular androgen, HRPCs still maintain some level of dependency to the AR pathway (10, 11, 26, 27) and our data also support this concept. However, of course, the retention of AR activity itself does not explain the more aggressive phenotype of clinical HRPCs, and apparently, the non-AR pathways should contribute to this clinical HRPC phenotype.

The list of up-regulated genes in HRPC (Table 1) included *ANLN* and *SNRPE* as well as *AR*. ANLN interacts with and activated RhoA and that this complex is likely to be essential for the growth-promoting pathway and aggressive features of lung cancers through phosphatidylinositol 3-kinase/Akt signaling (28), indicating that its overexpression in HRPCs can be involved with aggressive phenotype of clinical HRPCs. SNRPE may be involved with RNA splicing, but its function is unknown. Our siRNA experiments showed that overexpression of ANLN and SNRPE could play some important roles in prostate cancer cell viability and aggressive phenotype of HRPCs.

The list of the down-regulated genes in HRPC (Table 2) included *NR4A1*, *CYP27A1*, and *HLA-A* antigen. NR4A1 belongs to the steroid nuclear hormone receptor superfamily and its expression can cause apoptosis (29). NR4A1 expression is regulated by LH (30) and its down-regulation in HRPCs can reflect LH depletion in the patients under the treatment of LH-RH antagonist. CYP27A1 catalyses hydroxylations in the bioactivation of vitamin D3 (31). Epidemiologic evidence suggests an inverse relationship between prostate cancer and serum vitamin D levels (32), and active vitamin D3 inhibits growth and invasion of human prostate cancer cells (31). Down-regulation of NR4A1 and CYP27A1 can provide HRPC cells with some advantages for their survival and growth. Notably, HLA-A antigen, one of the MHC molecules, and many other HLA antigens (which were not listed in Table 2 because of their *P* value of 0.001–0.0001) were significantly down-regulated in clinical HRPCs, implicating that HRPC cells could acquire immunotolerance (33).

We attempted to identify the genes that were differentially expressed between HRPCs in metastatic site and those in the primary site (prostate). Because prostate cancer can preferentially metastasize to bone, several reports (34, 35) indicated that the microenvironment in bone marrow could promote prostate cancer growth and change their phenotype more aggressive. In comparing the gene expression patterns between metastatic tumors and primary tumors, it is critical to exclude the cells of the host organs of the metastatic tumors, and the expression profiles of bone metastasis of prostate cancer was very vulnerable

to contamination of bone marrow cells (26). In our study, the expression profiles of the microdissected cancer cells in bone metastasis was expected to reflect such inferences with the microenvironment in bone marrow and we did the supervised analysis using the expression profiles of 8 HRPC cells at bone metastatic lesions and 10 HRPC cells at the prostate. However, our analysis unexpectedly failed to distinguish them, and our supervised analysis did not clearly separate HRPC cells at the bone metastasis from those at the prostate. Taken together with the findings from the unsupervised hierarchical clustering analysis, our data indicated that the differences in expression patterns among the multiple metastatic loci derived from the individual patients were much smaller than the interindividual differences in the expression patterns.

In conclusion, our precise microarray analysis of clinical HRPC cells should provide useful information to understand the molecular mechanism of the hormone-refractory and more aggressive phenotypes of clinical HRPC and to identify molecular targets for the treatment of HRPCs.

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Expression of X-Linked Inhibitor of Apoptosis Protein Is a Strong Predictor of Human Prostate Cancer Recurrence

David B. Seligson,[1,5] Fumiya Hongo,[6] Sara Huerta-Yepez,[8] Yoichi Mizutani,[7] Tsuneharu Miki,[7] Hong Yu,[1] Steve Horvath,[2,3,5] David Chia,[1,5] Lee Goodglick,[1,5] and Benjamin Bonavida[4,5]

Abstract

Purpose: The X-linked inhibitor of apoptosis protein (XIAP) is associated with cell survival by blocking caspase-mediated apoptosis. We examined the expression patterns of XIAP with regard to human prostate cancer, predicting that XIAP status may predict cancer recurrence and/or clinical outcome.

Experimental Design: Immunohistochemistry was done on tissue microarrays constructed from 226 primary prostate cancer specimen. The protein expression distribution was examined across the spectrum of epithelial tissues and its association with standard clinicopathologic covariates and tumor recurrence was examined in 192 outcome-informative patients.

Results: The mean XIAP expression was significantly higher in prostate cancer compared with prostatic intraepithelial neoplasia (PIN), normal, and benign prostatic hyperplasia. We observed that XIAP is an independent predictor of tumor recurrence in multivariate Cox proportional hazards analysis in all patients as well as after substratifying by Gleason score. Interestingly, patients with high XIAP levels had a much lower probability of tumor recurrence than those with lower XIAP expression. Even patients with high-grade tumors who had higher XIAP levels had a lower risk of recurrence compared with any patient whose tumors express lower XIAP.

Conclusions: XIAP is expressed at higher levels in prostate cancers compared with matched normal tissues. High XIAP expression is strongly associated with a reduced risk of tumor recurrence and is not directly associated with Gleason score, tumor stage, capsular involvement, or preoperative prostate-specific antigen status, suggesting that it is a novel prognosticator and a potential target for prostate cancer diagnosis and therapy. Significantly, these findings provide important and extensive validation of previous results.

Prostate cancer is the most frequently diagnosed malignancy and ranks second among all cancers in men, with an estimated 232,090 new diagnoses and 30,350 deaths in the United States in 2005 (1). Most prostate cancers are clinically localized or regional upon diagnosis, and patients enjoy a 5-year survival rate approaching 100%.[9] Nonetheless, as evidence of the slow but steady nature of this disease, 30% to 40% will experience prostate-specific antigen (PSA) recurrence within 10 years following definitive surgery or radiation treatment (2). Patients with high risk or advanced disease on staging workup, or who have recurred, historically receive treatment with exogenous or endogenous androgen ablation, sometimes supplemented with chemotherapy and/or radiation (3). Unfortunately, progression of tumor cells to therapy resistance inevitably ensues, leaving few alternatives to care. As a result, the median survival in advanced disease is only 18 to 20 months, with an overall survival of 24 to 36 months.

Apoptosis (programmed cell death) is an important mechanism in tissue development, homeostasis, and response to stress factors. It relies on a concerted and tightly balanced signaling pathway involving pro- and antiapoptotic proteins. Dysregulation of apoptosis is a major contributor to tumorigenesis (4, 5), tumor growth (6), progression (4), metastases (7), and resistance to conventional therapies (8).

The mitochondrial pathway is activated by physiologic stress, including that induced by conventional cancer therapies, and is activated by p53 after DNA damage, ultimately resulting in increased mitochondrial membrane permeability and release of

Authors' Affiliations: Departments of [1]Pathology and Laboratory Medicine, [2]Biostatistics, [3]Human Genetics, [4]Microbiology, Immunology and Molecular Genetics, [5]Jonsson Comprehensive Cancer Center David Geffen School of Medicine at the University of California at Los Angeles, Los Angeles, California; [6]Department of Urology, Kyoto Second Red Hospital, [7]Department of Urology, Kyoto Prefectural University of Medicine, Kyoto, Japan; and [8]Unidad de Investigacion en Enfermedades Oncologicas, Hospital Infantil de Mexico, Federico Gomez Mexico D.F. Mexico

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Note: L. Goodglick and B. Bonavida contributed equally to this work.

Requests for reprints: Lee Goodglick, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, 10833 Le Conte Ave, Box 951747, 1P-430 CHS, Los Angeles, CA 90095-1747. Phone: 310-825-9134; E-mail: lgoodglick@mednet.ucla.edu.

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[9] American Cancer Society, http://www.cancer.org,