Prognostic Value of the Androgen Receptor and its Coactivators in Patients with D1 Prostate Cancer

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Abstract. *Background: Prostate cancer treated with androgen ablation eventually becomes resistant. Because the androgen receptor (AR) signaling axis affects disease progression, AR coactivator molecules could provide clinical prognostic value. This study investigates the association between AR coactivator molecules and clinical outcome measures in patients with prostate cancer. Patients and Methods: Expression levels of AR and its coactivators, SRC1, TIF2, and Her2/neu were determined by quantitative RT-PCR in 148 prostatectomy specimens. AR protein expression was determined by immunohistochemistry. The prognostic value of these expression levels on clinical outcomes was examined. Results: Increased gene and protein AR expression was not correlated with any of the clinical outcome measures. A non-monotonic correlation was observed between SRC1 and overall survival, as well as Her2/neu and time to prostate-specific PSA recurrence. Conclusion: Although no statistically significant relationships were found, the weak association between some clinical outcomes and two AR coactivators may help improve the current predictive nomogram for patients with prostate cancer.*

Prostate cancer is the most common cancer in American men over 65 years of age with more than 215,000 estimated new cases and more than 27,000 estimated deaths in 2007 (1). Approximately one third of patients treated with radical prostatectomy for localized prostate cancer will subsequently progress to metastatic disease (2). Although parameters, such as serum prostate-specific antigen (PSA), Gleason's score and tumor stage, can offer some risk stratification, more accurate prognostic markers for clinical outcome are necessary in order to determine the appropriate use of earlier and more aggressive adjuvant treatments.

Tumorigenesis and progression in prostate cancer require a functional androgen signaling axis, the components of which form the principal target of androgen ablation therapy commonly utilized to treat advanced disease. Despite an initial response in at least 80% of patients with metastatic disease, androgen ablation is palliative and disease progression eventually occurs (3). Although the mechanisms by which a tumor becomes hormone-refractory remain poorly understood, resistance to androgen ablation may not necessarily be due to loss of androgen sensitivity. Rather, it may develop as a consequence of a deregulated androgen signaling axis. The center of this concept is the androgen receptor (AR), which is reported to be expressed in essentially all metastatic tumors, including those that are hormone-refractory (4, 5). These studies show that AR amplification might be the cause of failure of endocrine therapy, but there is no conclusive evidence for this theory. Other studies have demonstrated that tumor recurrence and progression induce not only up-regulation of AR gene and protein, but also overexpression of AR coactivators, increased activation of mutated receptors by steroids and anti-androgens, and ligand-independent activation (6-8).

The overexpression of the p160 coactivators SRC1 (Steroid receptor coactivator-1) and TIF2 (Translation initiation factor eIF4A), observed in recurrent tumors from human prostate xenografts and clinical prostate cancer, increases AR transactivation capacity at physiological concentrations of non-classical ligands (9). Therefore, the functional characteristics of the AR can be modified simply by the overexpression of coregulators, making this mechanism a good candidate for functional selection under hormone ablation conditions.

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Key Words: Prostate cancer, androgen receptor, SRC1, TIF2, Her2/neu, prognosis, radical prostatectomy.

Another potentially important mechanism contributing to the failure of androgen ablation is ligand-independent activation of the AR through aberrant expression of growth factor or cytokine receptors (8), one of which is Her2/neu. Overexpression of HER2/neu, a transmembrane glycoprotein member of the epidermal growth factor receptor family, has been shown to enhance AR transactivation of various androgen-regulated genes in a ligand-independent manner and increase cell survival during androgen deprivation (10, 11). Thus, altered receptor-ligand interactions through amplification or mutation of the AR gene, modulation through interactions with coregulatory molecules, and/or ligand independent activation of the AR by growth factors and cytokines may be involved in prostate cancer progression under androgen withdrawal conditions.

In this study, the gene and protein expression levels of several molecular markers (AR, SRC1, TIF2, and HER2/neu) related to the androgen receptor signaling axis were compared with clinical outcomes in patients with lymph node positive prostate cancer (stage D1) treated with radical prostatectomy in order to determine whether the gene and protein expression levels have any prognostic value.

Patients and Methods

Tissue specimens. Between 1972 and 1999, 1,936 patients underwent radical retropubic prostatectomy and pelvic lymph node dissection for clinically organ-confined prostate adenocarcinoma at the USC/Norris Comprehensive Cancer Center. In this cohort, 235 patients were found to have metastases to the lymph nodes on final pathological examination (stage D1). Overall, 148 radical prostatectomy specimens were able to be retrieved from an IRB-approved tissue databank at the USC/Norris Comprehensive Cancer Center.

Immunohistochemistry. Formalin-fixed paraffin-embedded (FFPE) tissue blocks corresponding to the primary tumor were selected, from which 5-μm sections were cut into polylysine slides. Deparaffinization was performed with xylene and the tissue rehydrated in graded ethanol solutions and rinsed in tap water. The slides were buffered with 0.3% hydrogen peroxide and blocked with 20% fetal bovine serum, then incubated overnight at 4˚C with 1:250 fold dilution of a monoclonal mouse antibody against AR (Dako, Carpinteria, CA, USA). The tissue was then incubated for 1 hour at room temperature with the secondary antibody consisting of 1:1000 dilution of conjugated rabbit anti-mouse antibody (Dako). The slides were developed with diaminobenzidine tetrahydrochloride solution (Dako), lightly counterstained with hematoxylin and cover slipped before visualization.

Grading of the immunohistochemical staining. All slides were read and graded by two observers (R.M. and D.H.) blinded to the clinical outcomes. The AR protein expression was subjectively graded as weak or strong, depending on intensity of expression (0=no staining, 1=weak, 2=intermediate, and 3=strong), and the percentage of tissue showing immunoreactivity (positivity; 0%, 1-10%, >10%) was recorded in each case. Areas of benign epithelium within the slide served as the internal control in each case.

Microdissection. FFPE tumor specimens were cut into serial sections with a thickness of 10 μm. For the pathological diagnosis, one slide was stained with H&E and evaluated by a pathologist (D.H.). Other sections were stained with nuclear fast red (American Master Tech Scientific, Lodi, CA, USA) to enable visualization of histology. Laser captured microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) was performed in all the tumor samples to ensure that only tumor cells were dissected.

RNA isolation and cDNA synthesis. The tissue samples to be extracted were placed in 400 μl of 4 M guanidine isothiocyanate (4 M guanidinium isothiocyanate, 50 mM Tris-HCl (pH 7.5), 25 mM EDTA) (Invitrogen; #15577-018) containing 1 M dithiothreitol (DTT). The samples were heated to 92˚C for 30 min. Sodium acetate (2 M) (pH 4.0) and freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1) were used to extract the total RNA from the tissue suspensions. Glycogen and isopropanol were used for precipitation. The samples were air-dried for 15 min at room temperature. The pellet was re-suspended in 50 μl of 5 mM Tris. After RNA isolation, cDNA was prepared from each sample using random hexamers and M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase.

Reverse transcription PCR. Quantitation of AR, SRC1, TIF2, HER2/neu and an internal reference gene (β-actin) was carried out using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems, Foster City, CA, USA). The primers and probe sequences used are listed in Table I. The PCR reaction mixture consisted of 1200 nM of each primer, a 200 nM probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nM each of dATP, dCTP, dGTP, dTTP, 3.5 mM $MgCl_2$, and 1 Taqman Buffer A containing a reference dye, to a final volume of 20 μl (all reagents from PE Applied Biosystems). Cycling conditions were 50˚C for 2 min and 95˚C for 10 min, followed by 46 cycles at 95˚C for 15 sec and 60˚C for 1 min. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the genes of interest and the internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

Statistical methods. The outcomes used were overall survival, time to clinical recurrence and time to PSA recurrence (defined as a rise of PSA above the undetectable level of <0.3 ng/mL and verified by at least two consecutive increased PSA tests), *i.e.* calculated from the date of prostatectomy. For each outcome, data from those who did not experience the event were censored at the date of last follow-up. The outcomes among patients with different gene expression levels and AR protein expression were examined using both univariate log-rank test and log-rank test stratified by Gleason score or pre-operative PSA. The Pike estimates of relative hazard ratios were calculated with the use of observed and expected numbers of events as calculated in the log-rank test. Prior to analysis, all expression levels except for *Her2/neu* were categorized into 4 groups, with approximately equal number of patients in each group. *Her2/neu* expression was categorized into 3 groups because there were fewer distinct values. All *p*-values reported in the analyses are two-sided and values ≤0.05 were considered significant.

Table I. *Primers and probe sequences utilized.*

Gene	GenBank accession#	Forward primer (5'-3')	Reverse primer (5'-3')	Taqman® probe (5'-3')
β-Actin	NM_001101	TGAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT	ACCACCACGGCCGAGCGG
AR	NM_000044	TGTCGTCTTCGGAAATG TTATGA	GCCTCTCCTTCCTCCTGTAGTTT	AGATTACCAAGTTTCTTCAG CTTCCGGGCT
SRC1	NM_003743	TCCTCAGATGCAGCAGAATG	AAAGTTGGCCTCACCTTGG	CCATTCCTGCTCCTGGATACTGGA
TIF2	NM_006540	ACAGCCCTGTCACACCTGTT	CCCTGGTCGTGGGTTATTAAA	AACAGCACTGCGAATTTCACAGAGCA
HER2/neu	NM_004448.2	CTGAACTGGTGTATG CAGATTGC	TTCCGAGCGGCCAAGTC	TGTGTACGAGCCGCACATCCTCCA

Results

The clinical characteristics of the 148 patients are summarized in Table II.

Association between clinical outcome and AR protein expression. From the clinical samples collected, AR protein expression data from immunohistochemistry (IHC) was obtained on 75 primary prostate cancer, 42 normal prostate and 57 metastatic lymph node tissues. The prognostic value of AR protein expression in these tissues was looked at in regards to overall survival, time to clinical recurrence and time to PSA recurrence (Table III). AR positivity and intensity in primary prostate cancer and metastatic lymph nodes were not associated with any clinical outcome measures. However, patients with higher staining intensity for AR in normal tissue had significantly higher risks of PSA recurrence ($p=0.005$). Patients with higher staining positivity for AR in normal tissue had a statistically marginal higher risk of PSA recurrence ($p=0.070$). In the normal prostate, higher staining intensity or positivity for AR showed a trend towards clinical recurrence; this trend was not, however, statistically significant.

Association between clinical outcome and gene expression of AR and its coactivators. SRC1 mRNA levels in prostate cancer were significantly associated with overall survival ($p=0.031$) (Table IV). However, this association was not monotonic, with patients of the two intermediate levels having a smaller risk of dying than those of the lowest and highest group. The level of *Her2/neu* expression showed a non-monotonic association with time to PSA recurrence ($p=0.002$), with the intermediate group having the greatest risk. There was no association found between *TIF2* and *AR* expression levels with respect to time to PSA recurrence, time to clinical recurrence, or overall survival.

To exclude possible confounding effects of Gleason score and pre-surgery PSA, the log-rank test, stratified by either Gleason score or pre-surgery PSA, was performed for each

Table II. *Characteristics of patients with pathological stage D1 prostate cancer.*

	Number of patients (%)
Age at prostatectomy (years)	
<60	35 (23.7%)
60-65	44 (29.7%)
66-70	48 (32.4%)
>70	21 (14.2%)
Pre-operative PSA (ng/mL)	
<4	21 (14.2%)
4-9.9	35 (23.6%)
10-19.9	22 (14.9%)
≥20	28 (18.9%)
Missing	42 (28.4%)
Adjuvant hormonal therapy	
No	113 (76.4%)
Yes	35 (23.6%)
Adjuvant chemotherapy	
No	97 (65.5%)
Yes	51 (34.5%)
Adjuvant radiation therapy	
No	43 (29.1%)
Yes	105 (70.9%)
Pathologic stage (1992 AJCC)	
T2a/b	15 (10.1%)
T3a	32(21.6%)
T3b	91(61.5%)
T4a/b	10 (6.8%)
Gleason score	
4-6	18 (12.2%)
7	55 (37.2%)
8-10	75 (50.8%)
Margin status	
Negative	87 (58.8%)
Positive	61 (41.2%)
Seminal vesicle involvement	
No	47 (31.8%)
Yes	101 (68.2%)

Table III. *Association between clinical outcomes and AR protein expression measured by IHC in normal, tumor and LN tissues.*

		Overall survival			Time to clinical recurrence			Time to PSA recurrence		
	Expression	No. of patients	Relative hazard ratio	*P*-value	No. of patients	Relative hazard ratio	*P*-value	No. of patients	Relative hazard ratio	*P*-value
AR positivity*	0%	23	1.00	0.34	23	1.00	0.80	23	1.00	0.68
(Tumor)	1-10%	36	0.77		36	0.79		36	0.80	
	>10%	16	0.50		16	0.96		16	1.08	
AR intensity†	0	19	1.00	0.33	19	1.00	0.90	19	1.00	0.70
(Tumor)	1	28	0.53		28	1.11		28	1.15	
	>1	24	0.72		24	0.94		24	0.85	
AR positivity	0%	20	1.00	0.77	20	1.00	0.29	20	1.00	0.07
(Normal tissue)	1-10%	12	0.66		12	2.48		12	1.56	
	>10%	10	0.79		10	2.27		10	3.08	
AR intensity	0	20	1.00	0.57	20	1.00	0.16	20	1.00	0.005
(Normal tissue)	1	8	0.45		8	1.49		8	1.56	
	>1	13	0.78		13	2.90		13	3.08	
AR positivity	0%	16	1.00	0.99	16	1.00	0.60	16	1.00	0.66
(LN)	1-10%	30	0.93		30	1.54		30	1.46	
	>10%	10	0.91		10	1.79		10	1.23	
AR intensity	0	16	1.00	0.85	16	1.00	0.36	16	1.00	0.64
(LN)	1	20	0.84		20	1.36		20	1.50	
	>1	21	1.09		21	2.00		21	1.41	

*AR positivity, percentage of tissue showing immunoreactivity: 0%, 1-10%, >10%; †AR intensity, intensity of expression: 0=no staining, 1=weak, 2=intermediate, 3=strong. *P*-values were calculated for each tumor, normal tissue, and lymph node (LN) using the log-rank test.

Table IV. *Correlation between clinical outcomes and gene expression levels in prostatic tumor tissues.*

		Overall survival			Time to clinical recurrence			Time to PSA recurrence		
Gene	Expression relative to β-actin*	No. of patients	Relative hazard ratio	*P*-value	No. of patients	Relative hazard ratio	*P*-value	No. of patients	Relative hazard ratio	*P*-value†
AR	≤0.85	36	1.00	0.45	36	1.00	0.77	36	1.00	0.74
	0.85<AR≤1.3	36	0.90		36	0.79		36	1.05	
	1.3<AR≤2.0	33	1.53		33	1.10		33	1.19	
	>2.0	38	1.20		38	1.14		38	1.35	
SRC1	≤3.5	41	1.00	0.031	41	1.00	0.28	41	1.00	0.65
	3.5<SRC1≤4.5	33	0.67		33	0.89		33	0.85	
	4.5<SRC1≤6.3	38	0.92		38	0.62		38	1.26	
	>6.3	35	1.74		35	1.27		35	1.07	
TIF2	≤1.6	36	1.00	0.70	36	1.00	0.19	36	1.00	0.76
	1.6<TIF2≤2.4	39	0.71		39	0.50		39	1.19	
	2.4<TIF2≤3.5	35	0.92		35	0.61		35	0.96	
	>3.5	36	0.74		36	0.77		36	0.88	
Her2/neu	≤0.01	49	1.00	0.39	49	1.00	0.12	49	1.00	0.002
	0.01<Her2/neu≤0.04	50	1.54		50	1.94		50	2.44	
	>0.04	48	1.37		48	1.42		48	1.35	

*All expression levels, except for *Her2/neu*, were categorized into 4 groups, with approximately equal number of patients in each group; *Her2/neu* expression was categorized into 3 groups because there were few distinct values. †*P*-values were calculated using the log-rank test.

of the gene expressions. After controlling for Gleason score and pre-surgery PSA, the general patterns did not change.

Discussion

In patients with locally advanced prostate cancer undergoing primary treatment for the disease with either surgery or radiation therapy, several clinical and pathological indicators have been utilized to identify patients with a high risk of recurrence. Unfortunately, these clinical outcome predictors are not always accurate. The identification of novel molecular markers predictive of disease recurrence and overall survival might improve the currently used nomograms that are based on standard clinical and pathological features. The present study examines the prognostic value of protein and gene expression levels of AR and its coactivators with respect to clinical outcome measures in patients who had undergone prostatectomy for D1 disease.

Androgen receptor expression in prostate cancer is heterogeneous and conflicting data have been published regarding the correlations between AR levels and prostate cancer prognosis (12-14). The presence of the AR has been clearly demonstrated in primary prostate cancer tissues as well as in lymph node and bone metastases (4, 15, 16).

Some studies have shown that the concentrations of AR measured immunohistochemically might be a useful prognostic indicator for prostate cancer progression in patients treated with hormone ablation therapy (17-21), but this was not confirmed by others and remains controversial (20, 22-25). Studies examining the association between AR levels and clinical outcomes in patients undergoing radical prostatectomy also showed conflicting results. Li *et al.* demonstrated that elevated AR expression was associated with a shorter time to biochemical recurrence by studying prostate cancer tissues obtained from 640 prostatectomy samples (26). However, this observation was not confirmed by other studies. Our experiments failed to show a clear association between AR protein expression in tumor tissue and clinical outcome measures, including time to biochemical recurrence. One of the reasons for this discrepancy between the Li study and our findings could be due to the fact that some of our patients received adjuvant treatment, such as hormone therapy (23.6%), radiation therapy (70.9%) and chemotherapy (34.5%).

One mechanism of ligand-independent AR activation may be caused by increased expression of AR coactivators, such as SRC1, TIF2, and Her2/neu. SRC-1, the first nuclear receptor coactivator to be characterized, enhances AR transcription by directly influencing native histone acetyltransferase activity (27, 28). In addition, SRC-1 has been shown to up-regulate the transcriptional activity of the AR in a ligand-independent fashion. Another important coactivator of AR is TIF2. The significance of SRC-1 and TIF2 in progression towards androgen-independence is unknown, but their overexpression may provide a mechanism by which the AR function could be enhanced at low physiological androgen levels (9). The expression levels of SRC1 have been shown to be higher in cancer specimens with a higher grade or poor response to endocrine therapy than in those with a lower grade or good response to hormone ablation therapy (29). Interestingly, Linja *et al.* demonstrated that the expression of SRC1 is significantly lower in hormone-refractory prostate tumors than in untreated prostate tumors. The same group also found that gene amplification of *SRC1* in one of the prostate cancer xenograft models provided growth advantage (30). Our data indicate that *SRC1* gene expression was significantly associated with overall survival, with patients in the intermediate levels having a smaller risk of dying than those in the lowest and highest groups, and that *Her2/neu* was associated with PSA recurrence in a non-monotonic manner, with patients of the intermediate level having the greatest risk of developing PSA recurrence. Previously, *Her2/neu* examined in androgen-independent prostate cancer was shown to be associated with a significantly shorter survival time. This implicates *Her2/neu* in the progression of prostate cancer towards androgen-independence and more aggressive biology.

In conclusion, the addition of *SRC1* and *Her2/neu* expression levels might improve the current predictive value nomogram for patients with local or locally advanced prostate cancer. However, further studies on large cohorts of patients are needed to confirm this observation.

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Received November 12, 2007
Revised December 20, 2007
Accepted December 24, 2007