

Androgen Receptor Coactivators Lysine-Specific Histone Demethylase 1 and Four and a Half LIM Domain Protein 2 Predict Risk of Prostate Cancer Recurrence

Philip Kahl,¹ Lucia Gullotti,¹ Lukas Carl Heukamp,¹ Susanne Wolf,¹ Nicolaus Friedrichs,¹ Roland Vorreuther,³ Gerold Solleder,³ Patrick J. Bastian,² Jörg Ellinger,² Eric Metzger,³ Roland Schüle,⁴ and Reinhard Buettner¹

¹Institute of Pathology and ²Department of Urology, University Hospital Bonn Medical School; ³Department of Urology, Evangelische Kliniken Bonn GmbH, Bonn, Germany; and ⁴Center for Clinical Research, University of Freiburg Medical School, Freiburg, Germany

Abstract

Prostate cancer biology varies from locally confined tumors with low risk for relapse to tumors with high risk for progression even after radical prostatectomy. Currently, there are no reliable biomarkers to predict tumor relapse and poor clinical outcome. In this study, we correlated expression patterns of the androgen receptor (AR) coactivators lysine-specific histone demethylase 1 (LSD1) and four and a half LIM-domain protein 2 (FHL2), AR, Gleason score, Gleason grade, and p53 expression in clinically organ confined prostate cancers with relapse after radical prostatectomy. Our data reveal that high levels of LSD1, nuclear expression of the FHL2 coactivator, high Gleason score and grade, and very strong staining of nuclear p53 correlate significantly with relapse during follow-up. No correlation exists with relapse and the expression of AR and cytoplasmic expression of FHL2. To confirm these data, we did quantitative reverse transcription-PCR and Western blot analyses in a subset of tumor specimens. Consistently, both LSD1 mRNA and protein levels were significantly up-regulated in high-risk tumors. We previously identified LSD1 and FHL2 as nuclear cofactors interacting specifically with the AR in prostate cells and showed that both stimulate androgen-dependent gene transcription. Our present study suggests that LSD1 and nuclear FHL2 may serve as novel biomarkers predictive for prostate cancer with aggressive biology and point to a role of LSD1 and FHL2 in constitutive activation of AR-mediated growth signals. (Cancer Res 2006; 66(23): 11341-7)

Introduction

Prostate cancer represents the most frequent noncutaneous malignant disease in men worldwide and the second leading cause of death from malignant tumors (1, 2). The incidence is strongly related to age: Although prostate cancer is very rare below the age of 50 years, the incidence increases to ~1,150 cases per 100,000 males at the age of 80 years (3). In parallel, there is a significant increase in overall incidence. In the year 2000, there were 92,000 new cases and it is estimated that this figure will increase to 120,000 in the year 2020 (1).

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

P. Kahl and L. Gullotti contributed equally to this work.

Requests for reprints: Philip Kahl, Institute of Pathology, University Hospital Bonn Medical School, Sigmund-Freud-Strasse 25, D-53127 Bonn, Germany. Phone: 49-228-287-6488; E-mail: philip.kahl@ukb.uni-bonn.de.

©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-1570

The clinical outcome of prostate cancer is strongly related to its differentiation and malignancy grade (4). In particular, the Gleason scoring system makes use of the increasingly disturbed normal tissue architecture in high-grade carcinomas. However, Gleason grading is subject to interobserver variability (5) and requires expert opinion of experienced pathologists and, thus, has not proved to serve as an accurate predictor of clinical outcome when applied by different observers and laboratories (6). Although a large number of tumor suppressors and oncogenes have been identified and analyzed in prostate cancers, no surrogate markers are currently available that can be used to predict aggressive biology of prostate cancer and to adjust the extent and mode of therapy.

Similar to luminal prostate epithelial cells, the vast majority of prostate carcinomas express strong levels of androgen receptor (AR) and grow in an androgen-dependent manner. Hence, androgen ablation via castration and/or administration of small chemical inhibitors (e.g., luteinizing hormone-releasing hormone agonists or AR antagonists) is the most common treatment for advanced prostate cancer. However, after an initial response in the majority of cases, most tumors will ultimately progress to a hormone-refractory stage (7). Thus, constitutive activation of AR-mediated growth and subsequent androgen-independent receptor activation are important mechanisms involved in tumor progression. Likely, candidates involved in constitutive and hormone-independent receptor activation are transcriptional AR coactivators. Therefore, we aimed to identify such coactivators and to analyze their role in prostate cancer biology.

Screening for AR-interacting proteins previously identified lysine-specific histone demethylase 1 (LSD1; ref. 8) and four and a half LIM-domain protein 2 (FHL2; refs. 9, 10) as novel AR coactivators in prostate cancer cells. Initial observations indicated that LSD1 is strongly expressed in prostate cancers with high Gleason score. FHL2 expression occurs in a cytoplasmic manner in normal prostate glands, and the degree of nuclear translocation increases in less-differentiated cancer cells (10). In this study, we therefore systematically investigated LSD1 and FHL2 expression patterns in a cohort of 153 clinically organ confined tumors treated by radical prostatectomy and asked whether these patterns may serve as surrogate markers for aggressive biology and enhanced risk for tumor relapse.

Materials and Methods

Tissue microarrays. Tissue microarrays were prepared from formalin-fixed, paraffin-embedded tissue specimens of 153 prostate carcinomas selected from the archival files of the Institute of Pathology, University of Bonn Medical School. All tumor samples were surgically obtained from patients who had undergone radical retropubic prostatectomy in two

surgical centers between 1995 and 2002 for clinically organ confined prostate cancer (preoperative staging $\leq cT_2$, cN_0 , cM_0). Patients who had received prior hormonal therapy, chemotherapy, or radiation therapy were excluded from our study. All cases were reevaluated by a panel of experienced pathologists for histopathologic staging according to the Unio Internationale Contra Cancrum tumor-node-metastasis system (11), rescored according to the Gleason scoring system (2), and subsequently followed-up between 21 and 128 months (median 40.24 months). Three different tissue cores representing the lowest and highest Gleason grades within a single tumor were arrayed from formalin-fixed, paraffin-embedded tissue blocks using a manual device (Beecher Instruments, Sun Prairie, WI). Four-micrometer paraffin sections were cut from every tissue microarray and used for subsequent immunohistochemical analyses within 1 week.

Immunohistochemistry. Immunohistochemical staining was done as described previously (12) using the following antibodies and dilutions: α -LSD1 (8), 1:250; α -FHL2 (9), 1:250; AR (DAKO, clone AR441, Glostrup, Denmark), 1:75; and p53 (DAKO, clone DO-7), 1:250. Negative control reactions replacing the primary specific antibody by nonspecific immunoglobulin were done in all cases (shown in Supplementary Fig. S1). Immunostaining results for AR, LSD1, and nuclear and cytoplasmic FHL2 were evaluated considering only the carcinoma cells and using a semiquantitative scoring system as described (13). Briefly, the number of positive cells were counted and scaled (0, no positive cells; 1, 1-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; and 4, 76-100% positive cells). These scores were multiplied with an intensity scale (0, negative; 1, weak; 2, moderate; and 3, intensive staining). All slides were reviewed independently by two pathologists.

RNA isolation and quantitative reverse transcription-PCR. RNA was extracted from 10- μ m sections of formalin-fixed, paraffin-embedded tissue specimens by using the "Recover all" total nucleic acid isolation kit (Ambion, Austin, TX). Recovered RNA concentrations were measured using the Nanodrop 1000A spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription of the purified total RNA was carried out using the Omniscript reverse transcription kit with random hexamers for first-strand cDNA synthesis (Qiagen, Hilden, Germany).

LSD1 expression was quantitatively measured using the ABI 7900HT TaqMan instrument (Applied Biosystems, Foster City, CA). TaqMan reactions were done in 384-well plates according to the instructions of the manufacturer. Expression of LSD1 was measured in duplicates and normalized to 18S RNA. Primers for LSD1 and 18S RNA were labeled with 5'-FAM as a reporter and 3'-NFQ-1 as a quencher and purchased from Applied Biosystems.

Analysis of p53 mutation in exons 5 to 9. Tumor tissue for DNA extraction was marked on H&E-stained slides and microdissected from 10- μ m tissue sections. Extraction of genomic DNA from the tumor samples was done using the DNeasy Tissue kit (Qiagen) as described (14, 15). The following primer pairs were used for exon 5: forward 5'-TGCCGTGTTCCAGTTGCTTTATC-3' and reverse 5'-GCAATCAGTGAGGAATCAGAGGC-3'; for exon 6: forward 5'-AGCAGCTGGGCTGGAGAG-3' and reverse 5'-CCG-GAGGGCCACTGACAAC-3'; for exon 7: forward 5'-CCAAGCGCACTGG-CCTCA-3' and reverse 5'-AGCGGAAGCAGAGGCTGG-3'; for exons 8/9: forward 5'-CTGATTCCTTACTGCTC-3' and reverse 5'-CGGCATTTGAG-TGTTAGAC-3'. PCR was done in 50 μ L reactions containing template DNA, 2 μ mol/L of each primer, 0.25 units Platinum Taq DNA polymerase (Invitrogen, Karlsruhe, Germany), 5 μ L reaction buffer, 1.5 mmol/L $MgCl_2$, and 200 μ mol/L of each deoxynucleotide triphosphate. The PCR products were purified using polyethylene glycol precipitation. Template DNA concentrations for the cycle sequencing were estimated by agarose gel electrophoresis. Bidirectional DNA sequencing of the entire exons, including the corresponding exon-intron boundaries, was done with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) using the forward and reverse PCR primers. Cycle sequencing products were precipitated with 3M sodium acetate and analyzed on an ABI PRISM 310 capillary electrophoresis system (Applied Biosystems). The identity of the amplicon sequences was confirmed by database search.⁵

⁵ National Center for Biotechnology Information database: www.ncbi.nlm.nih.gov.

Western blot analyses. Protein lysates were extracted from homogenized specimens in 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mmol/L EDTA, and centrifuged at $13,000 \times g$ for 20 minutes at 4°C. Ten-microgram protein lysates were denatured in Laemmli buffer (Roth, Karlsruhe, Germany) at 90°C for 10 minutes, loaded on a 10% SDS-PAGE gel, and subjected to electrophoresis under reducing conditions. Proteins were transferred onto a polyvinylidene difluoride membrane (Roti-PVDF, Roth) using standard protocols. After blocking in 5% nonfat dry milk/PBST for 1 hour, the membranes were incubated for 1 hour with a polyclonal rabbit anti-LSD1 antibody (8), monoclonal anti-FHL2 antibody (9), polyclonal anti-AR (dilution 1:1,000; Cell Signalling), washed, incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:1,000; DAKO), and developed using enhanced chemiluminescence (Amersham, Little Chalfont, England). For quantitation, all blots were probed with an anti- β -actin antibody (dilution 1:5,000; DAKO) and the images were analyzed using the image processing and analysis program from the NIH. The β -actin signal was used to correct for unequal loading. Expression levels are indicated as the signal ratio of tumor samples compared with the corresponding normal tissue.

Groups and statistical analysis. Patients were allocated into two groups according to nonrelapse (group 1; $n = 112$) or relapse (group 2; $n = 41$). Relapse was defined as development of metastasis, histologically verified local recurrence, and/or prostate-specific antigen (PSA) relapse after primary treatment. PSA relapse was defined as a serum level above 0.2 ng/mL PSA confirmed by increasing PSA consecutively. Statistical analysis was done with the Mann-Whitney U test by using the SPSS 12.0 program (SPSS, Inc., Zürich, Switzerland) and by calculating the Spearman rank correlation coefficient (two-tailed). Informed consent was obtained from each patient and the study was approved by the University Ethical Committee (126/05). Cumulative relapse-free survival was presented as a Kaplan-Meier plot with χ^2 statistics.

Results and Discussion

For this study, we retrospectively analyzed expression patterns of the AR cofactors LSD1 and FHL2 within a prostate cancer collective from 153 patients. All patients were diagnosed by histologic analysis of biopsies and staged preclinically with organ-confined prostate cancer. Tissue processing of all specimens was done in our institution using identical procedures. Gleason score was determined independently by two pathologists and cases with discordant scores were revised by a panel review.

Patient data are summarized in Table 1. Follow-up was uneventful with respect to prostate cancer in 112 patients (group 1 = 73.2%) but 41 further patients were diagnosed with relapse within the follow-up observation period (group 2 = 26.8%). When further subgrouped according to Gleason score, relapses were

Table 1. Summary of clinical and histopathologic data

Group 1	$n = 112$ (nonrelapse)		
Group 2	$n = 41$ (relapse)		
Follow-up	21-128 mo (median 40.24 mo)		
Patients' age	49-80 y (median 68.5 y)		
Gleason score	2-7	8-10	
Total ($n = 153$)	$n = 94$	$n = 59$	
Relapse	$n = 20$	$n = 21$	$P < 0.037$
pT ₁	$n = 1$ (1.06 %)	$n = 0$	$P < 0.424$
pT ₂	$n = 58$ (61.7 %)	$n = 33$ (55.9%)	$P < 0.411$
pT ₃	$n = 31$ (32.9 %)	$n = 23$ (38.9%)	$P < 0.498$
pT ₄	$n = 4$ (4.2 %)	$n = 3$ (5.08%)	$P < 0.160$
pN ₀	$n = 90$ (95.7 %)	$n = 53$ (89.8%)	$P < 0.160$
pN ₁	$n = 4$ (4.2 %)	$n = 6$ (10.2%)	$P < 0.678$

Table 2. Statistical evaluation

Antibody	Expression pattern	Median group 1	Median group 2	Mean (CI) group 1	Mean (CI) group 2	<i>P</i>
LSD1	Nuclear	8	12	8.33 (7.6-8.9)	10.7 (10.0-11.4)	<0.001
FHL2	Cytoplasmic	2	2	2.01 (1.5-2.4)	3.04 (1.9-4.1)	<0.185
FHL2	Nuclear	1	2.5	2.59 (1.5-3.5)	5.85 (3.1-8.4)	<0.020
AR	Nuclear	3	4	3.50 (2.9-4.1)	5.29 (3.8-6.7)	<0.058
p53	Nuclear	0	0	0.20 (0.02-0.71)	1.39 (0.29-2.2)	<0.050
Gleason score		6	8	6.50 (6.1-6.8)	7.90 (7.4-8.3)	<0.001
Gleason grade		4	4	3.57 (3.3-3.7)	4.28 (4.0-4.5)	<0.001
LSD1 RT-PCR		88.2	90.1	84.1 (81.2-87.0)	87.7 (84.6-90.8)	<0.028

Abbreviation: CI, confidence interval.

significantly higher in the group of tumors with Gleason scores 8 to 10 than in patients with Gleason scores 2 to 7 ($P < 0.037$). Postoperative histologic analysis revealed tumor growth beyond the organ capsule (pT₃ or pT₄) in 61 cases (40.0%) and clinically occult lymph node metastases (pN₁) in 10 cases (6.6%). There were

slightly more understaged and metastasized carcinomas in the group of tumors with high Gleason score; however, this was not statistically significant (Table 1).

To assess differences in expression patterns of AR cofactors, we immunostained tissue microarrays for LSD1, FHL2, and AR.

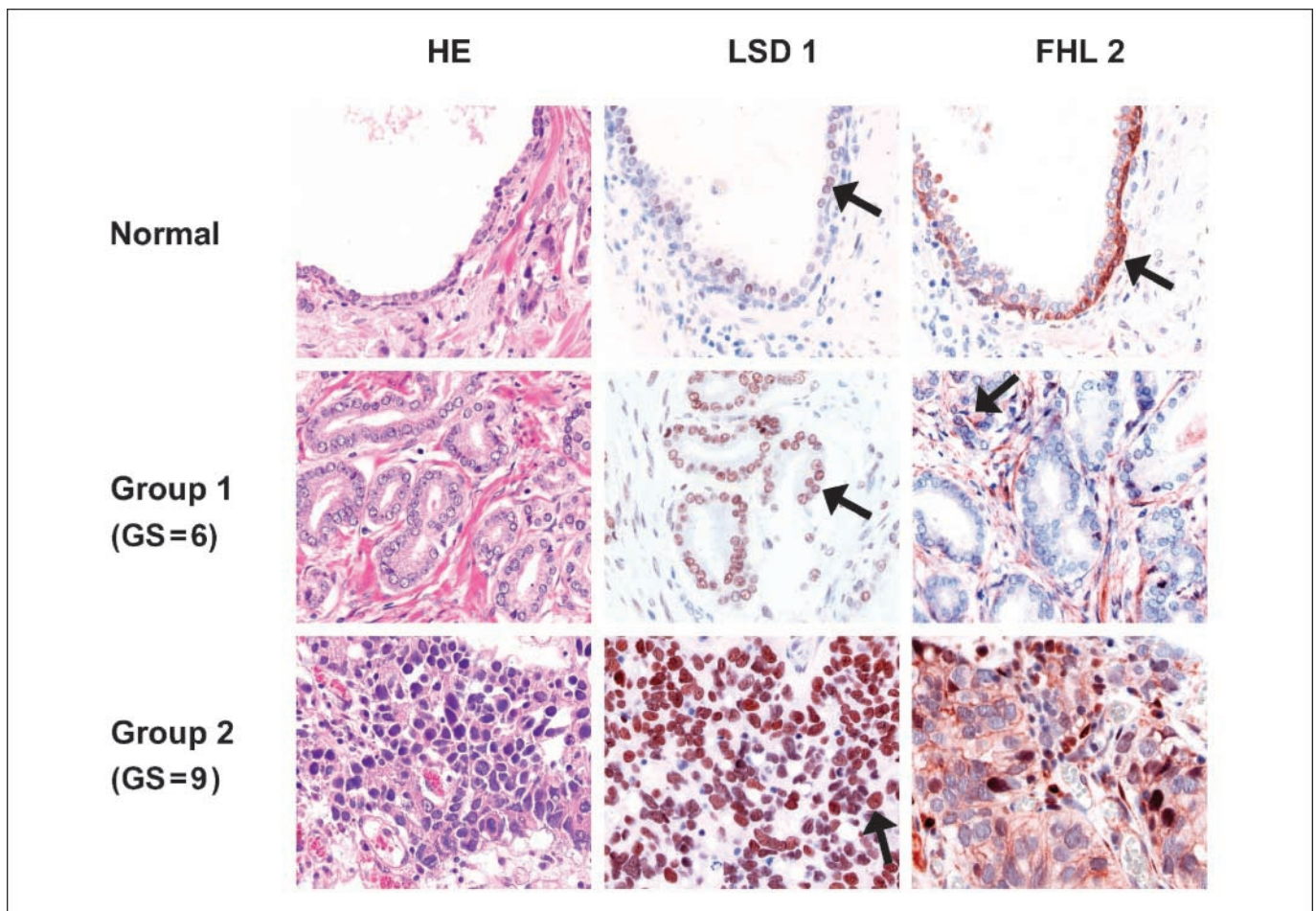


Figure 1. Representative immunohistochemical staining of LSD1 and FHL2 in normal prostate glands and tumors from patients without relapse [group 1, Gleason score (GS)=6] or with relapse (group 2, Gleason score 9). H&E staining shows standard morphology (magnification, $\times 400$). Arrows, nuclear LSD1 staining in luminal cells of normal prostate glands and in prostate carcinoma cells. Also, strong cytoplasmic signals in basal cells of normal glands and weak signals in group 1 carcinomas are depicted. Group 2 carcinoma cells show both strong nuclear signals and a combined plasmalemmal and cytoplasmic staining pattern.

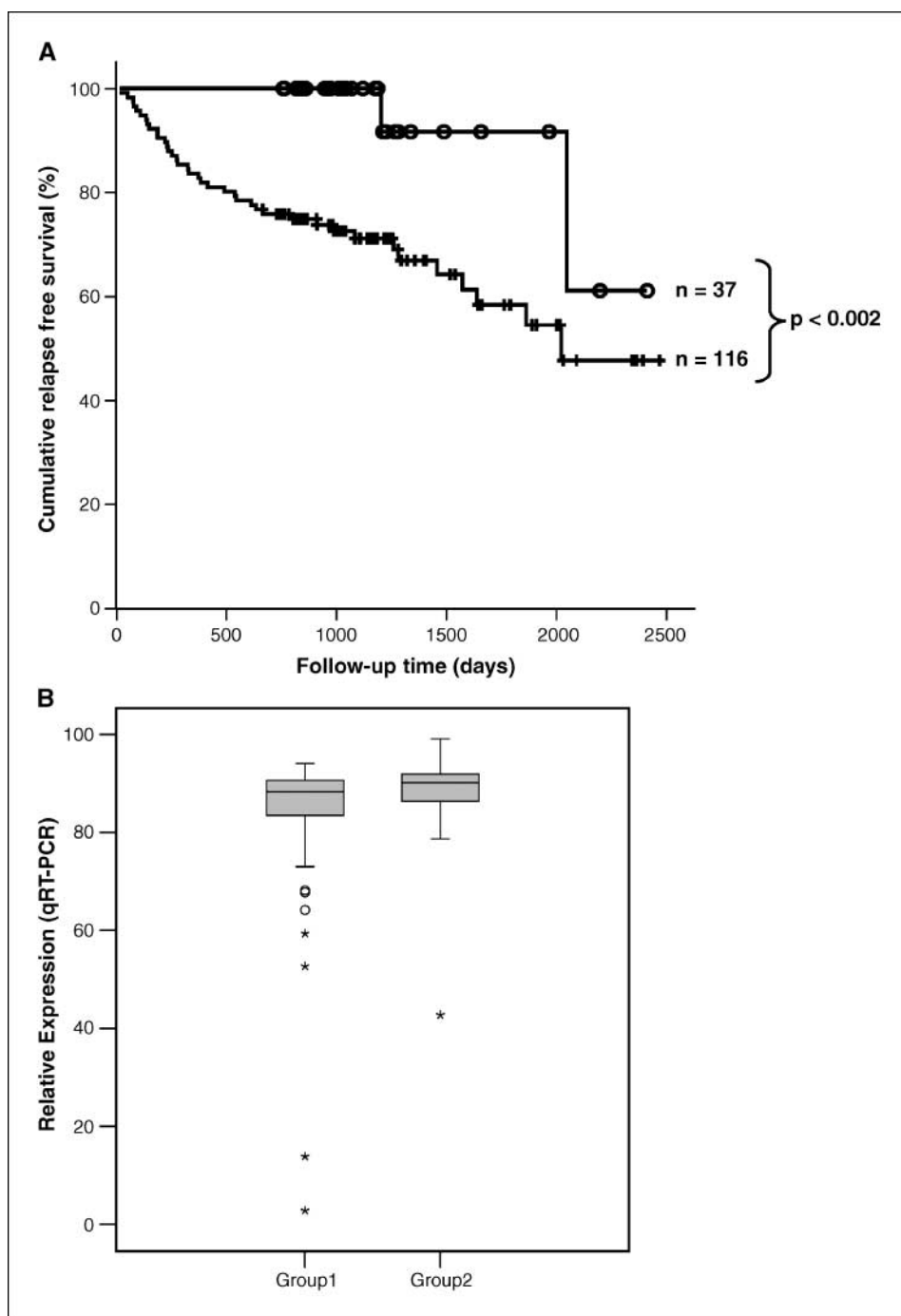


Figure 2. A, Kaplan-Meier plot showing cumulative relapse-free survival in cases with LSD1 scores of ≤ 8 (top) or > 8 . Log-rank (Mantel-Cox) analysis reveals significant difference between the curves ($P < 0.002$). B, box-plot showing arbitrary values of LSD1 mRNA expression (normalized to internal 18S RNA values) measured by quantitative RT-PCR (qRT-PCR). Mean values of group 1 and 2 are 84.1 (confidence interval, 81.2-87.0) versus 87.7 (confidence interval, 84.6-90.8), respectively.

Expression of p53 and Gleason score and grade were also analyzed to verify whether previously established variables for poor prognosis would discriminate the two groups. Nuclear LSD1, FHL2, or AR and cytoplasmic FHL2 signals were evaluated using a semi-quantitative score (13). Results summarized in Table 2 show that intense nuclear LSD1 ($P < 0.001$), nuclear FHL2 ($P < 0.02$), p53 ($P < 0.05$), Gleason score ($P < 0.001$), and Gleason grade ($P < 0.001$) discriminated group 1 patients (no relapse) from group 2 patients (relapse). In contrast, no significant differences were observed between the two groups concerning nuclear AR and cytoplasmic FHL2 staining.

More detailed histologic inspection revealed moderate nuclear LSD1 expression in luminal cells of normal prostate glands and

negative or very weak staining in basal glandular and stromal cells (see Fig. 1, arrows). Consistent with our recent study, significantly stronger staining was observed in carcinomas varying from 30% to 100% of all cancer cell nuclei. LSD1 immunoscores also correlated with Gleason scores as significantly more positive cancer cell nuclei and more intense staining were measured in carcinomas with Gleason scores 8 to 10 ($P < 0.001$). The Spearman rank correlation coefficient between LSD1 staining and all Gleason scores was 0.441. Typical staining patterns from normal prostate and tumors from groups 1 and 2 patients with low and high Gleason scores are shown in Fig. 1.

FHL2 staining was evaluated separately for cytoplasmic and nuclear staining. Previous studies identified FHL2 as a signal

transducer shuttling from the cytoplasm into the nucleus in response to activation of the small GTPase RhoA (10). Therefore, nuclear FHL2 indicates activation of the signaling pathway and the nuclear coregulatory activity. Consistent with our previous description, strong cytoplasmic FHL2 signals were present in basal cells of normal prostate glands but not in luminal cells (Fig. 1). As basal cells are abundantly present in normal prostate glands but absent in carcinoma glands, the overall expression level of cytoplasmic FHL2 was not up-regulated in carcinomas. However, carcinomas with high Gleason scores revealed significantly more nuclear staining in ~50% of the carcinoma cells than carcinomas with low Gleason scores. We previously verified, by cellular subfractionating and Western blotting, that the nuclear staining pattern of our FHL2 antibody indeed recognizes nuclear FHL translocated in response to RhoA activation (10).

Interestingly, AR staining did not differ significantly between groups 1 and 2 patients although there was a trend ($P < 0.058$) toward more intense staining in tumors of group 2 patients. Staining was observed in the nuclei of normal luminal cells and tended to be more intense in cancer cells. However, even in the carcinoma cells, proper staining intensity varied from very faint to intense patterns in 100% of the nuclei. Previous studies addressing the significance of AR staining as a predictive marker revealed controversial results (16, 17). Our data further suggest that AR immunostaining will not become a helpful biomarker to predict prostate cancer biology.

As immunohistochemical staining of LSD1 yielded semiquantitative results with some overlap between the two groups, we aimed to validate these data by correlating LSD1 levels with the respective time—from surgery to relapse of each case and by quantitating LSD1 mRNA expression levels using reverse transcription-

PCR (RT-PCR) analyses. Results of a Kaplan-Meier analysis shown in Fig. 2A indicated that tumors with LSD1 staining scores ≥ 8 significantly relapsed earlier and more frequently than tumors with staining scores < 8 . Statistical significance according to log-rank (Mantel-Cox) analysis was $P < 0.002$. Also, two-tailed Spearman rank correlation analysis indicated that high LSD1 immunoscores correlated with shorter disease-free survival within the relapse group (correlation coefficient, -0.517). In addition, measuring LSD1 mRNA expression by TaqMan quantitative RT-PCR yielded results very consistent with the immunohistochemical staining patterns (Fig. 2B). For these assays, we extracted total RNA from formalin-fixed and paraffin-embedded tissue specimens with $>50\%$ carcinoma cells and did TaqMan RT-PCRs using 18S RNA as an internal standard. The performance of the RT-PCR profile indicated that the quality of 126 RNA extracts (91 from group 1 and 35 from group 2) was suitable for PCR analysis. LSD1 expression was significantly higher in group 2 tumors than in group 1 tumors ($P < 0.028$), although there was considerable overlap between the two groups. The difference in RNA expression between groups 1 and 2 tumors was smaller than the difference in protein expression measured by immunohistochemical staining, most likely because RNA extracts from tumors always contained some portion of RNA from normal cells, such as stromal fibroblasts and lymphocytes, whereas immunostaining allowed to evaluate exclusively carcinoma cells.

In addition, we analyzed a small subset of groups 1 and 2 tumors by Western blots. Protein lysates of snap-frozen matched normal and carcinoma tissue samples with a tumor cell content of $>50\%$ were blotted and probed with LSD1, FHL2, AR, and for an internal standard with β -actin antisera. Results shown in Fig. 3 clearly indicated that LSD1 protein was more strongly expressed in all four

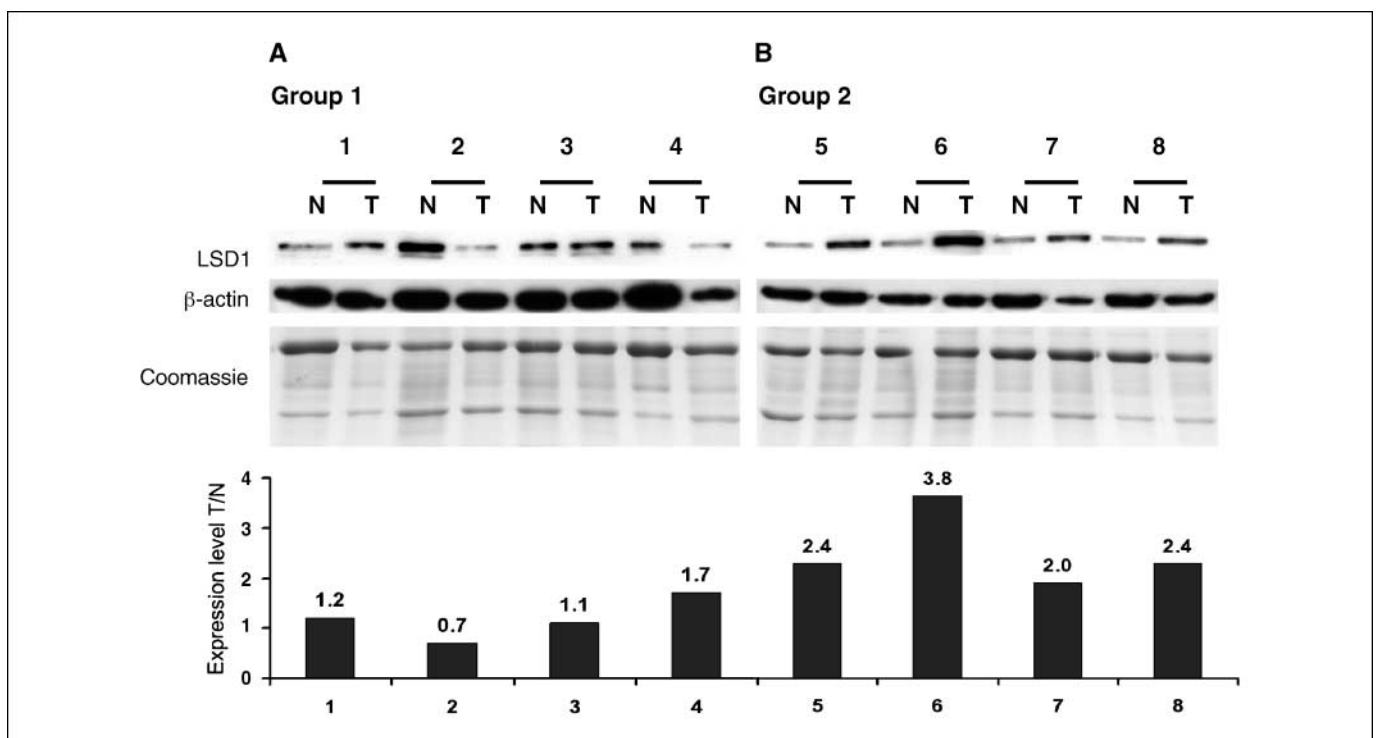


Figure 3. Western blot analysis of four different tumors from patients with no relapse (group 1, A) and patients with relapse (group 2, B) probed with α -LSD1 and α - β -actin antisera. Normal (N) and carcinoma (T) tissue recovered from the same prostate. *Bottom*, expression levels of LSD1 (normalized to β -actin) in the tumor sample relative to the corresponding adjacent normal tissue. Coomassie-stained gels used for Western blots are shown as control.

tumor extracts from group 2 patients (relapse). The ratio of LSD1 signals obtained from tumor and normal tissue was increased between 2.0- and 3.8-fold (Fig. 3, cases 5-8). In contrast, the ratio of normalized LSD1 signals from tumor and normal tissue of group 1 patients (no relapse) was only between 0.7 and 1.7 (Fig. 3, cases 1-4). Expression levels of total cellular FHL2 and of AR did not differ between the two tumor groups (Supplementary Fig. S2). As indicated above, total cellular FHL2 levels do not reflect Rho-activated FHL2 translocated into the nucleus, and therefore we did not expect to observe differences on Western blots.

Finally, we also tested whether p53 immunostaining differed between the two groups of patients. Sixteen carcinomas revealed strong nuclear staining in ~50% of the tumor cells. To control whether intense nuclear immunosignals resulted from pathogenic mutations in the DNA-binding domain of p53, we microdissected carcinoma cells from positive and negative cases and sequenced exons 5 to 9. As shown exemplarily in Fig. 4, all three p53-positive cases revealed pathogenic mutations known to disable DNA binding in contrast to three different p53-negative control cases that revealed wild-type sequences. Subgroup analysis revealed that p53-

immunopositive carcinomas were significantly more frequently detected in group 2 patients ($P < 0.05$).

In summary, our data indicate that group 2 patients (relapse) had carcinomas with significantly stronger nuclear LSD1, FHL2, and p53 immunoscores and with significantly higher Gleason score and Gleason grade. Also, tumors with high LSD1 protein and mRNA expression relapsed significantly earlier and more frequently than tumors with low LSD1 expression scores. Previous studies identified both LSD1 and nuclear FHL2 as transcriptional cofactors of AR-dependent gene activation (8, 10). Most prostate carcinomas grow in a hormone-dependent manner; therefore, overexpression of nuclear AR cofactors provide growth signals to prostate carcinoma cells. Interestingly, a recent study showed that global histone modification patterns predict risk of prostate cancer recurrence (18). We were able to show that LSD1 relieves repressive histone marks by demethylation of histone H3 at lysine 9 and thereby leads to derepression of AR (8). Taken together, these previous data and our present correlative study strongly suggest that LSD1 might be involved in epigenetic deregulation of prostate cancer and suggest a pathophysiologic link between histone

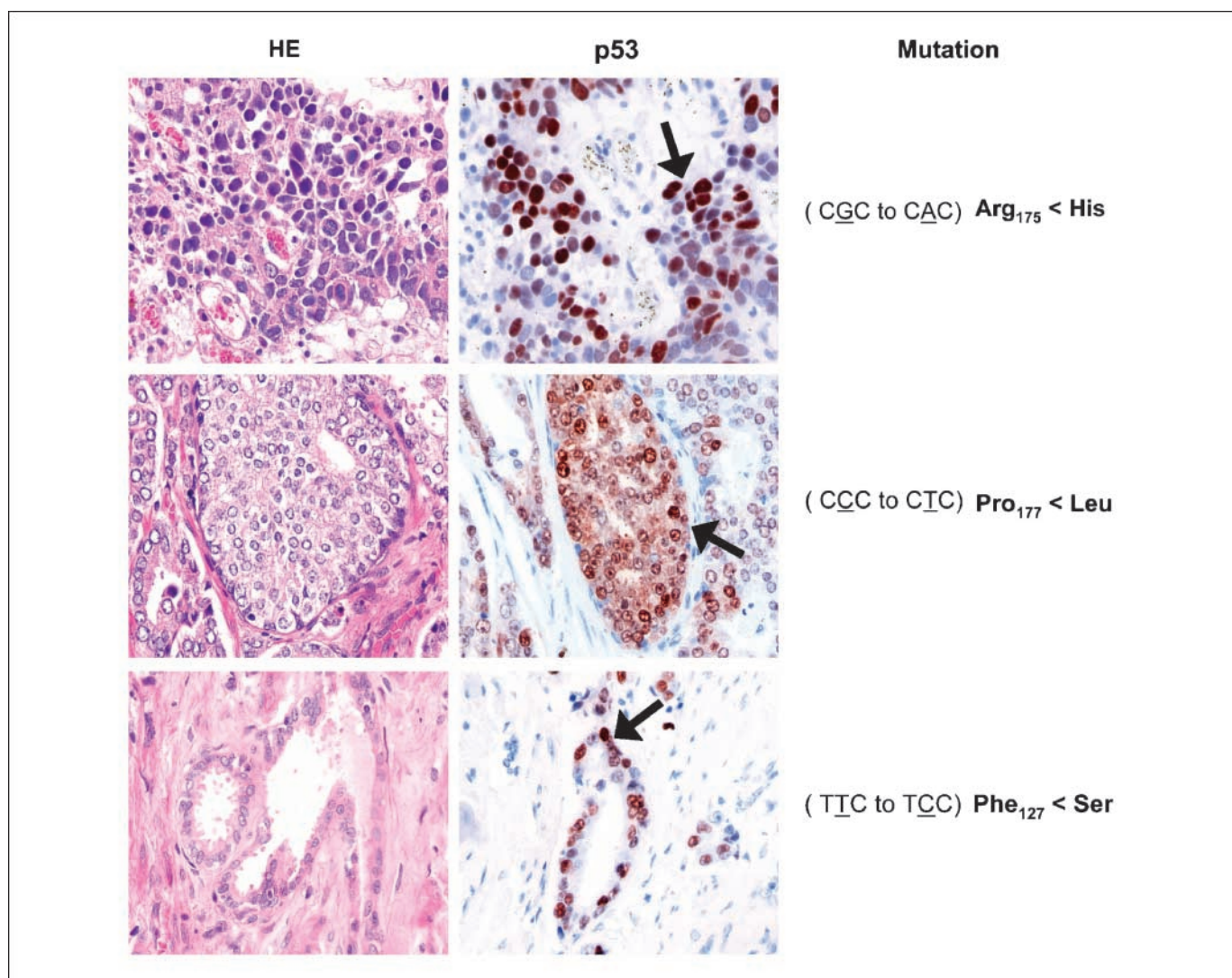


Figure 4. Nuclear stabilization of p53 in three representative prostate carcinomas. Sequencing genomic DNA recovered from the tumors revealed pathogenic p53 mutations in all three cases. Tumor morphology is shown as H&E staining (magnification, $\times 400$). Arrows, intense nuclear immunosignals.

demethylation, constitutive activation of androgen-dependent gene expression, and growth stimulation of prostate carcinomas.

Our data also strongly suggest that LSD1 might be an attractive novel therapeutic target for treatment of systemic prostate cancer. Notably, histone demethylation by LSD1 occurs through its monoamine oxidase activity, which is effectively inhibited by monoamine oxidase inhibitors such as pargyline. Indeed, we were able to previously show that pargyline treatment of the prostate cancer cell line LNCAP conferred dose-dependent growth inhibition. Therefore, epigenetic deregulation of gene expression resulting from overexpression of histone demethylases seems to be critically involved in prostate cancer progression and may provide a novel therapeutic target.

We also previously identified FHL2 as an AR transcriptional coactivator, which contains a strong, autonomous transactivation function and binds specifically to the AR *in vitro* and *in vivo* (10). Interestingly, we and others showed that FHL2 also binds to integrins and is found in focal adhesions in cultured cells (10, 19, 20). Nuclear translocation of FHL2 occurs in response to activation of the small GTPase RhoA by extracellular stimuli, such as the bioactive lipids lysophosphatidic acid or sphingosine-1-phosphate. Interestingly, prostate tumors overexpress Rho GTPases and display altered cellular localization of FHL2 concomitant with tumor dedifferentiation of prostate cancers and the amount of nuclear FHL2 correlated strongly with the Gleason grade (10). Taken together, we showed that stimulation of the Rho signaling pathway induces translocation of the coactivator FHL2 to the nucleus and transcriptional activation of FHL2- and AR-dependent reporter genes. Our present data indicate that activation of nuclear FHL2 signaling is linked to an aggressive biology and recurrence of prostate cancer.

A recent study comparing FHL2 mRNA expression in prostate cancer and normal prostate reported decreased expression levels of FHL2 mRNA in cancer tissue specimens (21). However, our

immunohistochemical data indicates, in agreement with our previous analysis of FHL2 expression (10), that very high levels of cytoplasmic FHL2 expression occur in basal cells of normal prostate glands. Because carcinomas represent the AR-positive differentiated luminal cell phenotype and are typically completely devoid of basal cell-like features, it is not unexpected that analysis of unfractionated carcinoma tissues falsely suggests down-regulation of FHL2. We therefore chose to study, in this and our previous study (10), nuclear translocation in carcinoma cells as a variable of Rho-activated FHL2 protein.

Importantly, immunostaining of both LSD1 and FHL2 provides novel biomarkers that can be assessed in parallel to p53 expression in preoperative biopsies of practically every prostate cancer. In contrast, Gleason scoring and grading are subject to considerable interobserver and interlaboratory variation. Given the huge effect of preoperative prostate biopsies for clinical management of prostate cancer, it is clear that additional biomarkers for accurate tumor evaluation and prediction are urgently needed and may be useful for preoperative decisions. With respect to the consistency and robustness of LSD1 and FHL2 immunoreactions, which are easily applicable to formalin-fixed small biopsies, we propose that these markers, together with p53, should be further studied as markers for prostate cancer with aggressive biology.

Acknowledgments

Received 5/3/2006; revised 8/14/2006; accepted 9/27/2006.

Grant support: Hans Messner-Stiftung and the Deutsche Krebshilfe (R. Schüle and R. Buettner); Graduiertenkolleg 804, Deutsche Forschungsgemeinschaft (L. Gullotti); and Bonfor (L.C. Heukamp).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Sebastian Fuß, Susanne Steiner, Inge Heim, Christiane Esch, Theresa Buhl, and Sandra Böhler for their expert technical assistance, and Gerrit Klemm for help with the artwork.

References

- Denmeade SR, Isaacs JT. A history of prostate cancer treatment. *Nat Rev Cancer* 2002;2:389–96.
- Hsing AW, Tsao L, DeVesa SS. International trends and patterns of prostate cancer incidence and mortality. *Int J Cancer* 2000;85:60–7.
- Waterbor JW, Bueschen AJ. Prostate cancer screening (United States). *Cancer Causes Control* 1995;6:267–74.
- Gleason DF, Mellinger GT. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol* 1974;111:58–64.
- Oyama T, Allsbrook WC, Jr., Kurokawa K, et al. A comparison of interobserver reproducibility of Gleason grading of prostatic carcinoma in Japan and the United States. *Arch Pathol Lab Med* 2005;129:1004–10.
- Bunting PS. Screening for prostate cancer with prostate-specific antigen: beware the biases. *Clin Chim Acta* 2002;315:71–97.
- Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem* 2004;91:483–90.
- Metzger E, Wissmann M, Yin N, et al. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 2005;437:436–9.
- Muller JM, Isele U, Metzger E, et al. FHL2, a novel tissue-specific coactivator of the androgen receptor. *EMBO J* 2000;19:359–69.
- Muller JM, Metzger E, Greschik H, et al. The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. *EMBO J* 2002;21:736–48.
- Schroder FH, Hermanek P, Denis L, Fair WR, Gospodarowicz MK, Pavone-Macaluso M. The TNM classification of prostate cancer. *Prostate Suppl* 1992;4:129–38.
- Pauls K, Schorle H, Jeske W, et al. Spatial expression of germ cell markers during maturation of human fetal male gonads: an immunohistochemical study. *Hum Reprod* 2006;21:397–404.
- Remmele W, Stegner HE. [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. *Pathologe* 1987;8:138–40.
- Wardelmann E, Hrychyk A, Merkelbach-Bruse S, et al. Association of platelet-derived growth factor receptor α mutations with gastric primary site and epithelioid or mixed cell morphology in gastrointestinal stromal tumors. *J Mol Diagn* 2004;6:197–204.
- Wardelmann E, Losen I, Hans V, et al. Deletion of Trp-557 and Lys-558 in the juxtamembrane domain of the c-kit protooncogene is associated with metastatic behavior of gastrointestinal stromal tumors. *Int J Cancer* 2003;106:887–95.
- Dunsmuir WD, Gillett CE, Meyer LC, et al. Molecular markers for predicting prostate cancer stage and survival. *BJU Int* 2000;86:869–78.
- Li R, Wheeler T, Dai H, Frolov A, Thompson T, Ayala G. High level of androgen receptor is associated with aggressive clinicopathologic features and decreased biochemical recurrence-free survival in prostate: cancer patients treated with radical prostatectomy. *Am J Surg Pathol* 2004;28:928–34.
- Seligson DB, Horvath S, Shi T, et al. Global histone modification patterns predict risk of prostate cancer recurrence. *Nature* 2005;435:1262–6.
- Li HY, Kotaka M, Kostin S, et al. Translocation of a human focal adhesion LIM-only protein, FHL2, during myofibrillogenesis and identification of LIM2 as the principal determinants of FHL2 focal adhesion localization. *Cell Motil Cytoskeleton* 2001;48:11–23.
- Wixler V, Geerts D, Laplantine E, et al. The LIM-only protein DRAL/FHL2 binds to the cytoplasmic domain of several α and β integrin chains and is recruited to adhesion complexes. *J Biol Chem* 2000;275:33669–78.
- Kinoshita M, Nakagawa T, Shimizu A, Katsuoaka Y. Differentially regulated androgen receptor transcriptional complex in prostate cancer compared with normal prostate. *Int J Urol* 2005;12:390–7.