Reduced Lecithin:Retinol Acyltransferase Expression Correlates with Increased Pathologic Tumor Stage in Bladder Cancer

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ABSTRACT

Purpose: Retinoids, which include vitamin A (retinol; ROL) and its derivatives, have been investigated in the treatment of bladder cancer. We have shown that expression of the enzyme lecithin:ROL acyltransferase (LRAT), which converts ROL to retinyl esters, is reduced in several human cancers. Here we evaluated expression of LRAT protein and mRNA in normal and malignant bladder tissue specimens from human patients. We also examined the effect of retinoids on LRAT expression in bladder cancer cell lines.

Experimental Design: We evaluated 49 bladder cancer specimens for LRAT protein expression using immunohistochemistry with affinity-purified antibodies to human LRAT. LRAT mRNA expression was assessed using reverse transcription-PCR in bladder specimens from an additional 16 patients. We examined the effect of retinoic acid and ROL on LRAT mRNA expression in five human bladder cancer cell lines.

Results: LRAT protein was detected throughout the nonneoplastic bladder epithelium in all of the specimens. In bladder tumors, LRAT protein expression was reduced compared with the nonneoplastic epithelium or was completely absent in 7 of 32 (21.9%) superficial tumors versus 16 of 17 (94.1%) invasive tumors (P < 0.001). All of the non-neoplastic bladder specimens tested (11 of 11) showed LRAT mRNA expression, compared with 5 of 8 (62%) superficial tumors and 0 of 5 (0%) invasive tumors (P = 0.001). Three of five human bladder cancer cell lines expressed LRAT mRNA independent of retinoid exposure, whereas in two cell lines LRAT mRNA expression was induced by retinoid treatment.

Conclusions: We report a significant reduction in LRAT expression in bladder cancer. Moreover, we demonstrate an inverse correlation of LRAT mRNA and protein expression with increasing tumor stage. These data suggest that loss of LRAT expression is associated with invasive bladder cancer.

INTRODUCTION

Bladder cancer is the fourth most common cancer among men in the United States. It has been estimated that there will be between 50,000 and 60,000 new cases of bladder cancer and ~12,000 deaths attributable to bladder cancer in 2003 (1). Transitional cell carcinoma (TCC), which refers to cancer arising from the transitional epithelium of the bladder, accounts for >90% of bladder cancers (2). Established risk factors for TCC include male gender, increasing age, and cigarette smoking (2). Approximately 80% of new cases of TCC are confined to the epithelium (Ta and carcinoma in situ) or lamina propria (T1) of the bladder at the time of diagnosis; these lesions are collectively referred to as superficial tumors. The remaining 20% of cases of TCC, which present with tumor involvement of the bladder muscle (T2), perivesical fat (T3), or adjacent organs (T4), are referred to as invasive tumors. Between 60% and 80% of superficial tumors will recur after treatment within 5 years, whereas 10–15% will progress to invasive disease.

Given the significant rate of recurrence and considerable morbidity associated with the progression of superficial bladder cancer, as well as the continued surveillance required in these patients, multiple preventative strategies have been attempted for this disease (2). One class of agents that has been investigated for potential chemopreventative and chemotherapeutic effects in TCC is the natural and synthetic analogs of vitamin A, a group of compounds known as retinoids (3). Vitamin A (retinol; ROL) levels have been shown to be significantly lower in the serum of patients with bladder cancer when compared with controls (4). Moreover, previous studies have demonstrated that retinoids were effective in preventing chemically induced bladder cancer in rats (5) and mice (6, 7). The effects of retinoids on growth inhibition of TCC have also been demonstrated in vitro in several human bladder cancer cell lines (8–10).

Clinical trials using retinoids for the chemoprevention of TCC have had various results. Several trials have failed to demonstrate a decrease in the recurrence rate of superficial tumors and have reported significant patient toxicities using
13-cis-retinoic acid (RA; Ref. 11) and etretinate (12), whereas other studies using etretinate have reported a decrease in tumor recurrence rates with acceptable patient side effects (13, 14). More recently, fenretinide, a synthetic derivative of all-trans-RA that has demonstrated activity as an inducer of apoptosis (10), has been shown to normalize cytology and decrease DNA aneuploidy in patients with superficial bladder cancer and abnormal cytology and/or DNA flow cytometry (15, 16).

The mechanisms by which retinoids regulate the growth and differentiation of cells have not been fully elucidated. The majority of actions of retinoids are thought to be mediated by two different families of nuclear receptors, RA receptors and retinoid X receptors, each consisting of three receptor types α, β, and γ (17). The expression profile of these receptors in bladder cancer has been explored previously in vitro for several human bladder carcinoma cell lines (9, 18). The RA receptors and retinoid X receptors act as ligand-activated transcription factors to regulate the expression of a number of retinoid-responsive genes (19).

ROL (vitamin A) can be metabolized into a variety of structurally related compounds, many of which have independent biological activity in various cell types (20, 21). For example, the esterification of ROL to retinyl esters is an important mechanism for maintaining tissue retinoids in a form that is easily stored and readily mobilized for additional metabolism (22). This process is catalyzed by the enzyme lecithin:ROL acyltransferase (LRAT), which has been cloned recently (22, 23). LRAT mRNA and protein expression have, in addition, been shown in select tissues to be regulated by exogenous retinoids (24). We have demonstrated previously that the metabolism of ROL to retinyl esters is greatly reduced in various human carcinoma cell lines, including prostate, kidney, skin, breast, and oral cavity, as compared with normal human cultured epithelial cell strains from these same human tissues (25–29). In addition, we have shown that LRAT levels were reduced in the tumor tissue from patients with renal cell carcinoma and prostate cancer compared with adjacent, normal tissue from these organs (25, 26, 30). These data suggest that human carcinoma cells are retinoid deficient relative to normal epithelial cells.

In this study, we evaluated the expression of LRAT protein and mRNA in normal and malignant human bladder tissue specimens. We additionally compared LRAT expression between superficial and invasive bladder tumors. In addition, we examined the expression of LRAT mRNA in bladder cancer cell lines and determined the retinoid sensitivity of LRAT expression by culturing the cells in the presence and absence of retinoic acid and ROL.

**MATERIALS AND METHODS**

**Cells and Culture Conditions.** Five well-characterized bladder cancer cell lines (HTB 1, HTB 2, HTB 3, HTB 9, and HT 1376) were purchased from American Type Culture Collection (Rockville, MD). The HTB 2 cell line is derived from a well-differentiated transitional cell papilloma, HTB 9 is from a moderately well-differentiated transitional cell carcinoma of the bladder, whereas HTB 1 and HT 1376 are derived from high-grade, invasive transitional cell carcinomas (31, 32). The HTB 3 cell line originated in an invasive squamous cell carcinoma of the bladder (31). Three independent experiments were performed for each cell line. Cultures were maintained at 37°C, 10% CO_2_ in DMEM supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% FCS. Cells were plated at 1 × 10^6 cells/plate on 100-mm tissue culture plates and allowed to attach for 24 h. To determine the effect of retinoid treatment on LRAT expression, cells were treated with 1 μM RA, 10 μM RA, or 1 μM ROL for 48 h. Untreated cells served as our control. RA and ROL were maintained as 1 × 10^{-7} M stocks dissolved in 100% ethanol, and all of the cells, including control cells, received an equal concentration of ethanol. All of the retinoid preparation and incubations were performed in the dark.

**Patient Tissue Collection.** We obtained 21 bladder specimens from 13 consecutive patients treated for TCC at New York Presbyterian Hospital between March 2003 and July 2003. Tissue was collected at the time of either transurethral resection or radical cystectomy. Samples were obtained from the visible tumor and, in 8 cases, from a grossly uninvolved portion of the bladder epithelium as well. Three additional bladder specimens were obtained from patients with prostate cancer undergoing radical retropubic prostatectomy. These 3 patients had no previous or current diagnosis of TCC, and thus these specimens were obtained as representative of normal bladder epithelium. Collection of tissue was approved by the Institutional Review Board of New York Presbyterian Hospital. Patient and tumor demographics are listed in Table 1. Tumors were staged on the basis of the final reports on the tissues submitted to the Department of Pathology, according to the American Joint Committee on Cancer-International Union Against Cancer Tumor-Node-Metastasis classification (33). Superficial tumors were considered to be tumors confined to the bladder epithelium or extending into the lamina propria only, whereas invasive tumors were characterized by tumor involvement of the muscularis propria or beyond. Tumors were graded using the 1998 World Health Organization/International Society of Urologic Pathology classification of papillary neoplasm of low malignant potential, low-grade urothelial carcinoma, and high-grade urothelial carcinoma (34). Tissue was frozen immediately after surgical resection at −70°C.

**RNA Isolation and Semiquantitative Reverse Transcription-PCR (RT-PCR).** Total cellular RNA was extracted from cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA). Patient tissue specimens were thawed and ground using a mortar and pestle in the presence of TRIzol. All of the subsequent steps were identical for the cell lines and patient samples. cDNA was prepared from 3-μg mRNA by reverse transcription with Superscript III Reverse Transcriptase (Invitrogen) at 55°C for 60 min. PCR was performed using gene-specific oligonucleotide primers. Primer pairs were designed to amplify cDNA sequences that in the genomic DNA cross an intron-exon boundary. Conditions for PCR on the patient samples consisted of 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. Each PCR contained 0.4 ng of each oligonucleotide primer, 2 μl of cDNA, 2.5 × 10^{-2} units Taq polymerase and accompanying 1× buffer (Invitrogen), 1.5 mM MgCl_2_, and 0.2 mM deoxyribonucleoside triphosphates. The LRAT (GenBank accession no. BC031053) primers 5’-ctc gcg cta cgg agc taa cat cc-3’ and 5’-gca gga agg gta tgt tat gat acc-3’ generated a 312-bp product. PCR amplification of β-actin was performed to confirm the
quality of the cDNA. A 379-bp fragment of \( \beta \)-actin (GenBank accession no. NM_001101) was generated using the sense primer 5'-gct cgt cgt cga caa cgg ctc-3' and the antisense primer 5'-gta cat ggc tgg ggt gtt gaa gg-3',

RT-PCR was performed on the patient samples without knowledge of the pathological diagnosis of the specimen. Conditions for RT-PCR on the cell lines were optimized for each primer pair to produce bands that were in linear range for \( \beta \)-actin and \( \beta \)-actin. For each PCR of patient samples and cell lines, an identical mix using water as the template (no cDNA) was included as a control for contamination. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The identity of the DNA product was confirmed by comparison of the PCR-amplified DNA to the predicted fragment size, as well as direct automated sequencing of the PCR product and comparison to the known DNA sequences of the target genes.

**Real-Time PCR Analysis.** Real-time PCR analysis was performed using a DNA Engine Opticon I system (MJ Research, Boston, MA) and SYBR green I Quantitect kit (Qiagen, Valencia, CA). Each reaction contained 1× Quantitect SYBR-Green I master mix, 0.3 \( \mu \)M of each primer, and 5 \( \mu \)l of diluted cDNA (described above) as template and was performed in low-profile tubes with optical caps (MJ Research). Real-time analysis used the primer pairs for \( LRAT \) and \( \beta \)-actin described above. PCR amplified fragments of the \( \beta \)-actin (5'-gct cgt cgt cga caa cgg ctc-3' and 5'-gca cag cct gga tag caa cgg-3') and \( LRAT \) (primers as described above) cDNAs were gel extracted (Qiagen) and cloned in pGEM-T (Promega, Madison, WI) for use as template standards. The identity of each cloned fragment was confirmed by direct automated DNA sequencing.

A standard curve was generated for each gene by amplifying known amounts of each cDNA target at the same time as the patient samples. The threshold cycle was set at 10 times the SD above the mean baseline emission for the first 10 cycles. The threshold cycle values showed linear correlation with relative DNA input for both the \( LRAT \) and \( \beta \)-actin primer pairs used. Expression of \( \beta \)-actin was used as an internal reference. Reaction conditions were 95°C for 3 min to activate the polymerase; followed by 50 cycles of 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 20 s; fluorescence was read after each cycle at 80°C. Real-time analysis was performed in triplicate for each cDNA sample. Negative control PCR reactions using water in place of template were performed for each experiment.

**Immunohistochemistry.** Paraffin-embedded tissue specimens were obtained from 49 consecutive patients treated for bladder cancer at New York Presbyterian Hospital between July 2002 and June 2003. All of the cases were urothelial carcinoma/transitional cell carcinoma. Patient and tumor demographics are listed in Table 1. A representative tissue block from each tumor was selected for immunohistochemical analysis. The analysis of these tissues was approved by the Institutional Review Board of New York Presbyterian Hospital.

Tissue localization of LRAT protein was performed as described previously (30) using an affinity-purified, polyclonal rabbit antihuman LRAT antibody (22). Tissue sections (5 \( \mu \)m) were cut from blocks, deparaffinized in Histo-Clear (National Diagnostics, Atlanta, GA), and rehydrated in a graded series of ethanol. Antigen retrieval was performed by heat with Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) in a pressure cooker for 2 min. A 3% solution of \( H_2 O_2 \) was used to quench the endogenous peroxidase activity (15-min incubation). Slides were then incubated in primary rabbit anti-LRAT antibody diluted 1:250 in 1.5% goat serum for 1 h at room temperature and treated with horseradish peroxidase polymer conjugate (Picture plus kit; Zymed, San Francisco, CA) for 30 min. Color was developed with 3,3′-diaminobenzidine chromogen/substrate followed by counterstaining with hematoxylin (Vector Laboratories). The negative control tumor sections were treated identically to all of the other sections except that 1.5% normal goat serum was used in place of the primary antibody.

**Table 1** Demographics of patients included in RT-PCR* and immunohistochemical analysis for LRAT

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Patients analyzed for LRAT mRNA expression via RT-PCR (n = 16)</th>
<th>Sex</th>
<th>Patients analyzed for LRAT protein expression via immunohistochemistry (n = 49)</th>
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<tbody>
<tr>
<td>Mean</td>
<td>65</td>
<td>Male</td>
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<tr>
<td>Range</td>
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<td>Female</td>
<td>43–88</td>
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<tr>
<td>Pathologic tumor stage</td>
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<tr>
<td>T0 (benign bladder)</td>
<td>3</td>
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<tr>
<td>Ta</td>
<td>5</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>CIS</td>
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<td></td>
</tr>
<tr>
<td>T1</td>
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<td></td>
</tr>
<tr>
<td>T2</td>
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<td>10</td>
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</tr>
<tr>
<td>T3</td>
<td>0</td>
<td>7</td>
<td></td>
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<tr>
<td>Total no. superficial tumors (Ta + CIS + T1)</td>
<td>8</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Total no. invasive tumors (T2 + T3)</td>
<td>5</td>
<td>17</td>
<td></td>
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<tr>
<td>Pathologic tumor grade</td>
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<td>0</td>
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<td>Low grade</td>
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<tr>
<td>High grade</td>
<td>9</td>
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</tbody>
</table>

*RT-PCR, reverse transcription-PCR; LRAT, lecithin:retinol acyltransferase; CIS, carcinoma in situ.
The immunohistochemical expression of LRAT protein was scored in a semiquantitative fashion by a single pathologist (S. K. T.). Specimens were graded according to the intensity of staining within the tumor compared with the intensity of staining of the adjacent, benign bladder epithelium. Tumors were classified as 0 (no staining), 1+ (weak staining), 2+ (distinct staining, but weaker than the staining in benign bladder), and 3+ (staining equal to that in the benign tissue). The immunohistochemistry procedure and sample evaluation were performed independently and blinded to pathological tumor stage.

**Statistical Analysis.** LRAT mRNA expression in the benign bladder specimens obtained from patients was compared with expression in superficial and invasive tumor samples using the $\chi^2$ test. LRAT immunohistochemical staining in superficial and invasive patient tumors was also compared using the $\chi^2$ test. Statistical analysis was performed by the Biostatistics Core Facility of Weill Medical College of Cornell University.

**RESULTS**

**Immunohistochemical Analysis of LRAT Protein Expression in Nontumor Bladder Epithelium and in Superficial and Invasive Bladder Tumors.** The expression of LRAT protein in the human bladder has not been examined previously. We evaluated 49 specimens, consisting of 32 superficial and 17 invasive tumors. Tumors and adjacent, benign bladder epithelium were examined for the presence of LRAT staining (Fig. 1). We found that the non-neoplastic bladder exhibited diffuse, cytoplasmic staining uniformly throughout the cell layers of the bladder epithelium (Fig. 1A). Except for inflammatory cells, other structures in the bladder, including the lamina propria and muscularis propria, did not stain (Fig. 1A). Because of the strong LRAT expression in the benign bladder epithelium, we used this staining for the grading of the immunostaining in the tumors and as a positive internal control. None of the negative controls showed any staining either in the tumor or in the benign adjacent bladder epithelium (Fig. 1B).

The LRAT immunoreactivity in individual tumors was generally uniform. In several of the invasive tumors, a difference in the staining intensity between the muscle invasive component of the tumor and the superficial, noninvasive segment of tumor was observed. In such cases, LRAT immunoreactivity was graded according to the staining intensity of the invasive component. All 19 cases of Ta tumors (Fig. 1C) and carcinoma in situ (lesions confined to the bladder epithelium) demonstrated LRAT immunoreactivity. Staining intensity was equivalent to the benign bladder epithelium (3+) in 18 of 19 of these tumors (e.g., Fig. 1C) and was 2+ in 1 case. Twelve of the 13 T1 tumors (Fig. 1D) showed evidence of LRAT immunoreactivity, 7 lesions with 3+ intensity expression, 3 with 2+ expression (i.e., Fig. 1D) that was weaker than that in the adjacent, benign bladder (arrows in Fig. 1D), and 2 tumors with weak (1+) staining. Five of the 10 T2 tumors showed weak (1+) staining (Fig. 1E) compared with the adjacent, nonneoplastic bladder (Fig. 1E, arrows), whereas 1 T2 lesion possessed 3+ immunoreactivity, 2 tumors demonstrated 2+ staining, and 2 lesions did not express LRAT. Four of the 7 T3 lesions (Fig. 1F) similarly lacked any evidence of LRAT expression. The summarized distribution of LRAT immunostaining in the superficial and invasive tumors is shown in Fig. 2. A significantly greater percentage of superficial (96.9%) than invasive (64.7%) tumors demonstrated evidence (1+, 2+, or 3+ staining) of LRAT expression ($P = 0.002$). Moreover, in the invasive tumors that did express LRAT, the level of LRAT immunoreactivity (reflected by the intensity of staining for the LRAT antibody) was weaker, as significantly fewer invasive tumors (35.3%) possessed 2+ or 3+ intensity staining than did superficial tumors (90.6%; $P < 0.0001$).

An independent statistical correlation of LRAT expression with tumor grade was not performed, as no tumors were classified as low malignant potential, whereas all 9 of the lesions classified as low-grade urothelial carcinoma were Ta stage tumors. The remaining (40) bladder tumors, which included T1, T2, T3, and carcinoma in situ lesions, were high-grade urothelial carcinoma.

**RT-PCR Analysis of LRAT mRNA Expression in Benign Bladder Tissue versus Superficial and Invasive Bladder Tumors.** Twenty-four fresh bladder specimens from 16 patients were evaluated. In 8 patients, both tumor and adjacent nonmucosal bladder were obtained. Thirteen of the 24 specimens were from bladder tumors: 8 of the tumors studied were superficial, and 5 were invasive. The pathological stage distribution of the tumors is listed in Table 1. Eleven nonneoplastic bladder specimens were evaluated, consisting of the 8 samples, mentioned above, from normal-appearing bladder epithelium adjacent to TCC tumors, and 3 specimens obtained from patients undergoing radical prostatectomy, who had no previous or current diagnosis of TCC.

Results from a representative semiquantitative RT-PCR analysis of the patient samples are shown in Fig. 3. Differences in LRAT mRNA expression were demonstrated both between the benign and tumor bladder tissue from the same patient, as well as between superficial and invasive tumors in separate patients. The LRAT mRNA expression for the 24 samples tested is summarized in Table 2. All (11 of 11) of the nonneoplastic bladder specimens evaluated demonstrated LRAT mRNA expression, compared with 5 of 8 (62%) of superficial tumors and 0 of 5 (0%) of invasive tumors ($P = 0.001$).

No independent correlation between tumor grade and LRAT mRNA expression was detected; 4 of 13 tumors were classified as low grade, and all 4 were superficial lesions. LRAT mRNA was detected in 3 of 4 of these tumors, versus 2 of 4 high-grade superficial tumors. All 5 of the invasive lesions studied, 0 of 5 of which, as described above, expressed LRAT mRNA, were classified as high-grade tumors.

The tumors analyzed for LRAT expression with RT-PCR were from consecutive patients treated over a different time interval (March 2003 to July 2003) than the tumors analyzed with immunohistochemistry for LRAT (July 2002 to June 2003). In addition, the tumors that we analyzed with immunohistochemistry were paraffin-embedded specimens obtained retrospectively from archival tissue in our Department of Pathology. Thus, mRNA analysis with RT-PCR of these paraffin-embedded specimens was not possible. As a result, most of the tumors analyzed with immunohistochemistry were distinct from the tumors analyzed with RT-PCR. However, because there was an overlap in the time intervals of tissue collection, 7 samples from 4 patients analyzed for LRAT mRNA expression with RT-PCR were also evaluated using immu-
LRAT protein and mRNA expression correlated in each of these 7 specimens.

Specifically, in 2 patients with Ta superficial lesions in which LRAT mRNA was detected by RT-PCR in both the tumor and nontumor sample, 3+ immunostaining was noted in both the tumor and adjacent, nontumor tissue. In addition, no LRAT immunohistochemical staining was found in a T3 lesion, which similarly had no detectable LRAT mRNA. One patient

Fig. 1 Immunohistochemistry of lecithin:retinol acyltransferase (LRAT) expression in formalin-fixed, paraffin-embedded sections from bladder tumors of various stages. Samples were incubated with the LRAT antibody, followed by horseradish peroxidase polymer conjugate. Hematoxylin was used as the counterstain. A, LRAT protein expression in the nonneoplastic bladder epithelium. We found that the nontumor bladder exhibited diffuse cytoplasmic staining uniformly throughout the cell layers of the bladder epithelium. Because of the strong LRAT expression in the benign bladder epithelium, we used this staining for grading of the immunostaining in the tumors and as a positive internal control. B, negative control bladder specimen. Negative control sections were treated identically to other sections except that 1.5% normal goat serum was used in place of the primary antibody; none of the negative controls showed any staining either in the tumor or in the benign adjacent bladder epithelium. C, 3+ intensity (equivalent intensity to the benign bladder epithelium) expression of LRAT in a Ta superficial tumor. D, 2+ immunostaining with LRAT antibody in a T1 (lamina propria invasion) superficial tumor. Grade 3+ staining is seen in adjacent, benign epithelium (arrows). E, LRAT protein expression is decreased in more invasive bladder tumors: 1+ staining in a T2 (muscle invasive) tumor, with grade 3+ staining in adjacent benign epithelium (arrows). Also, note stronger intensity staining in the superficial (involving the lamina propria) component of the tumor, indicating a gradient of expression within the tumor (♀). F, absent (0) immunoreactivity in a separate muscle invasive specimen. Images A and B reflect ×200 magnification, whereas C–F represent ×100.
with a T2 tumor, in which LRAT mRNA was not detected in the tumor but was detected in the nontumor specimen, demonstrated 1+ immunostaining in the tumor and 3+ in the nontumor bladder tissue.

**Real-Time PCR Analysis of LRAT mRNA Expression in Superficial and Invasive Bladder Tumors.** Real-time RT-PCR was performed on the RNA isolated from the tumor and adjacent nontumor bladder tissue from 4 patients, 2 with invasive and 2 with superficial tumors. Expression of LRAT mRNA was greatly reduced or absent in the tumor samples from patients with both superficial and invasive tumors compared with adjacent, nonneoplastic bladder (Fig. 4), supporting our findings with semiquantitative PCR (Fig. 3; Table 2).

**Retinoid Regulation of LRAT mRNA Expression in Bladder Cancer Cell Lines.** Steady-state and retinoid-treated LRAT mRNA expression was assessed by RT-PCR in five bladder cancer cell lines (Fig. 5). LRAT mRNA was detected in the untreated cells of 3 (HTB 2, HTB 3, and HTB 9) lines; expression of LRAT mRNA did not change in any of these cell lines after 48 h of treatment with either RA or ROL. In contrast, LRAT mRNA was increased in HTB 1 cells, which exhibited low levels of constitutive LRAT expression, by RA and by ROL treatment. RA regulation of LRAT mRNA in HTB 1 cells was demonstrated to be dose-dependent, as treatment with 1 μM RA resulted in greater mRNA expression than treatment with 10 pm RA. LRAT transcripts were induced by exposure to RA or ROL in HT 1376 cells, which did not demonstrate LRAT mRNA expression in untreated cells. A similar dose-dependent response to RA was seen in these cells. Of interest, the lowest basal LRAT mRNA expression occurred in the TCC cell lines with the most aggressive phenotype, as both HTB 1 and HT 1376 are derived from high-grade, invasive TCC tumors, whereas HTB 2, HTB 3, and HTB 9 are derived from a well-differentiated papilloma, a squamous cell carcinoma, and a moderately well-differentiated TCC, respectively. Nevertheless, LRAT mRNA expression in the high-grade TCC cell lines was retinoid responsive.

**DISCUSSION**

Retinyl esters are the predominant metabolites of vitamin A (ROL) in most epithelial cells and tissues. Not only is conversion of ROL to retinyl esters believed to regulate cellular uptake and storage of vitamin A, but, in addition, retinyl esters may serve as an internal source of ROL for the synthesis of RA. Retinoic acid, in turn, is a ligand for the nuclear RA receptors, which bind to RA response elements in target genes, thereby regulating gene transcription (35–38). Two enzymes have been reported to catalyze retinyl ester synthesis, LRAT and acyl-CoA:ROL acyltransferase. LRAT levels are reduced in many types of carcinoma specimens and cell lines (25–29). Given the stated importance of retinyl esterification, then, it is likely that reduced expression of LRAT would result in reduced levels of RA, and thereby render the affected cells at least in part functionally retinoid deficient.

In this study, we investigated the expression of LRAT in

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**Fig. 2** Distribution of lecithin:retinol acyltransferase (LRAT) protein expression in superficial (*n* = 32) and invasive (*n* = 17) tumors. Superficial tumors included pathological stages Ta, carcinoma *in situ*, and T1, whereas invasive tumors included T2 and T3 lesions. The grade of LRAT immunostaining is shown as 0 (no detectable staining), 1+ (weak staining), 2+ (distinct staining, but weaker than the staining in benign bladder), and 3+ (staining equal to or greater than that in the benign tissue). □, superficial; ■, invasive.

**Fig. 3** LRAT mRNA expression in superficial and invasive tumors compared with adjacent, nonneoplastic bladder samples from the same patient. Results from 7 patients are shown in whom tumor tissue as well as adjacent, benign bladder tissue was obtained, and RNA isolated and analyzed for LRAT mRNA expression using reverse transcription-PCR. *Lanes* 1–7 demonstrate LRAT mRNA expression in the nonneoplastic specimens, whereas *lanes* 8–14 represent LRAT mRNA expression in the corresponding tumor samples; for example, *lane* 8 represents the tumor sample from the same patient whose benign bladder tissue is evaluated in *lane* 1, and *lane* 9 represents the same patient as *lane* 2. LRAT mRNA expression in 4 superficial tumors and 3 invasive tumors are compared here. Amplification using PCR primers specific for β-actin confirmed the integrity of the cDNAs used. PCR for both LRAT and β-actin expression consisted of 35 cycles for all patient samples tested.

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We showed that the expression of LRAT mRNA and protein levels are decreased in TCC of the bladder with increasing pathological tumor stage. These data extend our previous research, which has shown that several types of human carcinomas, including breast, head and neck, skin, prostate, and kidney, exhibit major alterations in LRAT transcripts and a reduced ability to accumulate retinyl esters (25–29). We have also recently demonstrated differential LRAT protein expression in subtypes of renal cell carcinoma; specifically, LRAT expression was found to be higher in renal tumors, such as chromophobe renal cell carcinoma and renal oncocytoma, with an indolent biological behavior (30). However, the mechanism(s) by which the expression of LRAT mRNA and protein is altered in cancer cells is not yet understood.

In fact, the genetic and epigenetic events responsible for malignant transformation of normal bladder epithelium and tumor progression in TCC have been the subjects of multiple recent investigations (2, 39–42). For example, the mutation and/or overexpression of epidermal growth factor receptor (43, 44), erbB-2 receptor, hepatocyte growth factor and its receptor hepatocyte growth factor receptor/c-Met (45, 46), and H-Ras (47–49) have been associated with bladder cancer and, in several reports, have been correlated with tumor aggressiveness. Other studies have demonstrated genetic alterations, including point mutations and gene deletions, which result in inactivation of the tumor suppressor genes such as p53 and Rb in bladder cancer (39, 41, 42). Promoter hypermethylation, which causes the transcriptional silencing of genes in TCC (50, 51), has also been observed. We are currently investigating the potential mechanisms for the loss of LRAT expression, associated with progressive bladder carcinogenesis, which we observed.

The prognostic value of LRAT expression in bladder tumors similarly cannot be addressed by this study. A separate investigation of the potential impact of LRAT expression on clinical outcome of patients with bladder cancer is in progress. Of particular interest will be a difference in outcomes for patients with similar pathological stage tumors, which exhibit differential LRAT expression. From a potential therapeutic standpoint, LRAT expression was restored here after retinoid treatment in two TCC cell lines, which demonstrated minimal or absent basal LRAT mRNA expression. Retinoid-induced mRNA expression of several retinoid-responsive genes has been shown previously in human bladder cancer cell lines (18); LRAT activity, meanwhile, has been demonstrated in the livers of the human urinary bladder. We found that LRAT protein expression in the nonneoplastic bladder was uniform throughout all of the cell layers of the bladder epithelium. Next, we demonstrated that both LRAT mRNA and protein levels are decreased in TCC compared with adjacent, noncancerous bladder tissue. In addition, we showed that LRAT mRNA and protein expression was detected in each sample. Expression of LRAT in bladder tumor samples is presented as the percentage of LRAT expression detected in the adjacent, nonneoplastic bladder. Reverse transcription-PCR was performed in triplicate for each cDNA sample, and mean data are presented. *, lecithin:retinol acyltransferase (LRAT) expression was not detected under the conditions used. □, #1 noncancer; □, #1 invasive tumor; ■, #2 nontumor; ■, #2 invasive tumor; □, #3 nontumor; □, #3 superficial tumor; □, #4 nontumor; □, #4 superficial tumor; *, not detected.

### Table 2 Summary of RT-PCR for LRAT mRNA expression in patient specimens

<table>
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<tr>
<th>Pathology of specimen</th>
<th>No. cases</th>
<th>No. expressing LRAT (%)</th>
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<tr>
<td>Nonneoplastic bladder</td>
<td>11</td>
<td>11 (100)</td>
</tr>
<tr>
<td>Superficial bladder tumor</td>
<td>8</td>
<td>5 (62)</td>
</tr>
<tr>
<td>Invasive bladder tumor</td>
<td>5</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*RT-PCR, reverse transcription-PCR; LRAT, lecithin:retinol acyltransferase.*
vitamin A-deficient rats after retinoid exposure (24). Given the known impact of retinoids on cell growth and differentiation (19), along with the importance of LRAT in maintaining retinoid levels in tissues (25, 26), restoration of LRAT expression may provide one molecular mechanism for the effects of retinoids in human bladder cancer, as reviewed previously (3, 52, 53).

Retinoids and the structurally related compounds, retinamides, have been shown to inhibit cell proliferation, stimulate cell-cycle arrest, and induce apoptosis in human bladder cancer cell lines (8, 10, 54). Animal studies evaluating the chemopreventive effects of retinoids have found that a range of synthetic and naturally occurring retinoids inhibit chemically induced bladder carcinogenesis in rats and mice (5–7, 55). Furthermore, in human studies, serum vitamin A levels were shown to be lower in patients with bladder cancer (4), whereas a reduced incidence of bladder cancer was noted in patients with a high dietary intake of vitamin A or carotenoids (56, 57).

Prospective trials using retinoids in the treatment of bladder cancer have investigated the secondary chemopreventive effects of various retinoids in superficial TCC. An early study treated 30 patients with a previous history of superficial TCC with etretinate, a synthetic retinoid derivative, and reported a significant decrease in the recurrence rate of superficial tumors after a mean of 17.6 months of treatment (13). Although a second, contemporary study, which treated etretinate at the same dosage in 73 patients with a history of superficial TCC, found no effect of the agent on preventing tumor recurrence (12), a third prospective randomized, multicenter trial with etretinate demonstrated that the number of recurrent tumors, as well as the mean interval between recurrences, were significantly lower in etretinate-treated patients (14).

Studies involving 13-cis-RA for the chemoprevention of superficial bladder cancer have met with largely disappointing results, as Prout et al. (11) found no effect of the agent on tumor recurrence rate. In contrast, studies using the synthetic retinoid derivative fenretinide [N-(4-hydroxyphenyl)retinamide] in patients with superficial bladder cancer have reported more favorable results, albeit with different endpoints. That is, Decensi et al. (15) demonstrated a normalization of positive or suspicious urinary cytology after fenretinide treatment in patients with a history of superficial TCC. The same investigators, using DNA flow cytometry analysis of bladder washings, have also found a significant decrease in the proportion of patients with DNA aneuploidy in bladder-washed cells after fenretinide treatment (15, 16).

In summary, our data show a significant reduction in LRAT expression in bladder cancer compared with nonneoplastic bladder epithelium and an inverse correlation of LRAT expression with increasing tumor stage. We suggest that restoration of LRAT expression may be a reasonable strategy for TCC prevention and/or therapy. Future studies will further define the role of retinoid metabolism in the development and progression of bladder cancer.

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Reduced Lecithin: Retinol Acyltransferase Expression Correlates with Increased Pathologic Tumor Stage in Bladder Cancer


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