Mechanistic Analysis of the Role of BLCA-4 in Bladder Cancer Pathobiology

Julie M. Myers-Irvin, Thu-Suong Van Le, and Robert H. Getzenberg

Departments of Urology, Pathology, and Pharmacology, University of Pittsburgh and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Abstract

Analysis of alterations in nuclear structure associated with bladder cancer has revealed specific changes associated with the disease. This includes the identification of six bladder cancer-specific proteins and the successful development of urine-based immunoassays for the detection of two of these biomarkers, BLCA-1 and BLCA-4. The purpose of this study is to examine the functional aspects of BLCA-4 and its potential role in bladder cancer pathobiology. Sequence analysis of BLCA-4 reveals that it is a member of the ETS transcription factor family and that it seems to associate with transcription factors. To examine the effects of this protein, the gene encoding BLCA-4 was stably transfected into human urothelial cells. BLCA-4 expression was confirmed by both PCR and Western blot analysis. BLCA-4 overexpressing clones exhibit a 4.3-fold greater proliferation rate than vector only controls or untransfected cells. Microarray analysis comparing gene expression patterns between overexpressing clones and vector only controls revealed that numerous genes were up-regulated in cells that overexpress BLCA-4. Up-regulated genes included interleukin-1α (IL-1α), IL-8, thrombomodulin, and the protein expression of these genes was confirmed by immunoblot analysis. This information has provided a potential model of BLCA-4 action. Overexpression of BLCA-4 seems to increase the growth rate in cells and also causes cells to express a more tumorigenic phenotype. (Cancer Res 2005; 65(16): 7145-50)

Introduction

Bladder cancer is the fourth most commonly diagnosed cancer in men and the 10th most commonly diagnosed in women, according to the 2005 American Cancer Center statistics. It is estimated that 63,210 new cases of bladder cancer will be diagnosed in the United States and nearly 13,180 total deaths are predicted as a result of this disease (1). More than 90% of bladder cancers are transitional cell carcinomas (2). At the time of diagnosis, about 75% of patients have superficial bladder cancer, with tumors confined to the mucosa or lamina propria. Superficial bladder cancer has a very high rate of recurrence with up to 70% of patients presenting with additional tumors after treatment. Of these recurrent tumors, 10% to 30% will present with grade and stage progression (3).

The most common symptom seen in ~80% of bladder cancer patients is hematuria, or the presence of blood in the urine. However, this symptom is often indicative of diseases other than bladder cancer, such as kidney stones or bladder infections. It is reported that >90% of patients that present with hematuria do not in fact have bladder cancer. Other symptoms of the disease include painful or difficult urination, increased frequency of urination, or abdominal pain (4). However, these symptoms can be symptoms of many other diseases. Because there are few diagnostic symptoms of bladder cancer, a sensitive and specific test is needed to detect this disease. Currently, bladder cancer is most often diagnosed using urine cytology and cystoscopy. Cytology involves microscopic examination of cells in the urine. This test has a very high specificity but lacks sensitivity and requires a trained pathologist for review (2).

Our lab has previously identified six nuclear matrix proteins that are specifically expressed in bladder cancer (5). Nuclear matrix proteins are the scaffold of the nucleus and have a variety of functions, many of which are implicated in tumorigenesis. A few of the functions of the nuclear matrix include DNA organization, stabilization, and orientation during replication, determination of nuclear morphology, organization of gene regulatory complexes, and synthesis of RNA (6). Sequence data has been obtained from BLCA-4 and it has established that this protein is detectable in both the tissue and urine of individuals with bladder cancer. An immunoassay using BLCA-4 and a study of a large cohort of patients with bladder cancer, other benign urologic conditions, other cancer types, and normal controls, showed a specificity of 95% and a sensitivity of 89% (4).

The gene that encodes BLCA-4 has been identified and sequenced and has homology with the ELK-3 gene, a member of the ETS transcription factor family. Preliminary work has been published demonstrating that BLCA-4 confers a growth advantage in T24 cells transfected with the gene encoding BLCA-4 when compared with vector only controls. In addition, it has been shown that BLCA-4 can interact with the ETS binding sequence as well as several transcription factors, including AP-1, AP-2, NFATC, NF-E1, and NF-E2 (4).

The purpose of this study is to further study the functional aspects of the BLCA-4 gene and its potential role in bladder cancer pathobiology. To accomplish this, the gene encoding BLCA-4 was transfected into human urothelial cells. These cells were used to study cell growth as well as examine gene expression of the transfected and untransfected cells via microarray. A few genes found to be up-regulated in the transfected cells were selected to further confirm their expression via immunoblot analysis.

Materials and Methods

Transfection of BLCA-4. BLCA-4 cDNA (in pZeo-SV2 vector for constitutive expression) was transfected into HUC, a nontumorigenic human uroepithelial cell line transformed with SV40 (American Type Culture Collection, Manassas, VA), using the LipofectAMINE Plus kit (Invitrogen, Carlsbad, CA). HUC cells were plated to ~70% confluence in 6-well plates. Each well of a 6-well plate was transfected with increasing...
amounts of DNA (0.5, 1, 2, 3, or 4 μg BLCA-4 cDNA or 1 μg vector only). Cells were selected with zeocin 48 hours after transfection to create stable transfectants. Each clone was isolated using clonal discs and transferred to a 24-well plate. Once confluent, the transfected cells were gradually transferred to a 6-well plate, then T25 cm² and subsequently T75 cm² flasks to isolate cell lysates or RNA. Cell lysates were isolated from confluent T75 flasks as described previously (7). RNA was isolated from cells using the RNeasy midi kit (Qiagen, Valencia, CA).

Cell proliferation assay. Two thousand cells of each clone were plated in eight wells of a 96-well plate on day 1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done on days 3, 4, 5, and 6. The assay was done by making 5 μg/mL of thiazolyl blue tetrazolium bromide (Sigma-Aldrich, St. Louis, MO) in 1× PBS. Fifty microliters of MTT solution were added to each well and incubated for 3 hours. The MTT solution was aspirated off and 200 μL of DMSO were added to each well. The absorbance was read immediately at 595 nm.

PCR. One-microgram RNA isolated from transfected cells was reverse transcribed to cDNA using the Reflection RT kit (Active Motif, Carlsbad, CA). PCR amplifications were done using 2 μg cDNA, 10 μmol/L forward and reverse primer, water up to 25 μL, and 25 μL of RedMix Plus 1.5 mol/L MgCl₂ (Gene Choice, Frederick, MD). PCR was done using the following conditions: 94°C for 3 minutes, denaturation at 92°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 2 minutes and 30 seconds, for a total of 30 cycles. Degenerate primers consisted of BLCA-4 forward primer 5'-CCNCGRFTYAYGTTGATATTC-3' and BLCA-4 reverse primer 5'-GNTAYGARGAYATHTGCARAA-3' (Invitrogen). The glyceraldehyde-3-phosphate dehydrogenase control primers consisted of a 753-bp fragment using the forward primer 5'-GACCTCAACTCATGGTCTACATG-3' and the reverse primer 5'-TGTGCTGTGTTGAAGTCAGGGACAG-3'.

Microarray analysis. RNA was hybridized to the Affymetrix U133 Plus 2.0 (Whole Genome) chip, and raw data collection was done as previously described (8).

Immunoblot analysis. Immunoblot analysis was done as previously described (9). Briefly, 10 μg of cell lysates were loaded and separated by SDS-PAGE. After transfer and blocking, the membranes were incubated at room temperature with anti-interleukin-8 (IL-8, 1:100), anti-thrombomodulin (1:500), or anti-IL1-α (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. A secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA; 1:10,000), was incubated at room temperature for 1 hour. Detection was achieved by a chemiluminescence reaction using the enhanced chemiluminescence Immunoblot kit (Amersham Life Sciences, Piscataway, NJ).

Results

To begin to understand the function of BLCA-4 in bladder tumorigenesis, the gene encoding this protein was stably transfected into a human immortalized cell line. Transfected cells were treated with zeocin 48 hours after transfection and clones were picked 2 weeks later. The expression of BLCA-4 in several clones was analyzed by PCR using BLCA-4 gene-specific primers. Four BLCA-4-overexpressing clones and one vector only clone were selected for future studies based on their BLCA-4 expression (Fig. 1A). Clones 1 and 21 highly overexpress BLCA-4, whereas clones 9 and 11 express BLCA-4 mRNA at lower levels. Immunoblots were done to examine BLCA-4 protein expression in the clones verified as expressing BLCA-4 at the message level. Clone 21 expresses the highest level of BLCA-4 protein, and a lower quantity is produced by clone 1 (Fig. 1B). To examine the role of BLCA-4 in cell proliferation, an MTT assay was done on the transfected and control cells. Each time point for this assay is an average absorbance from eight wells, and this assay is representative of multiple experiments. Due to variations in absorbance readings, the values between experiments could not be combined, but the growth patterns were similar between experiments. The clones transfected with the gene encoding for BLCA-4 had a 4.3-fold greater proliferation rate than vector only controls or untransfected HUC cells (Fig. 1C). Cell counting assays were also done to confirm the growth advantage in the transfected clones, and comparable growth patterns were observed (data not shown).

To examine alterations in gene expression that may be occurring as a result of BLCA-4 expression, gene expression patterns were compared between overexpressing and control lines. The BLCA-4-expressing clone 21 was chosen for analysis due to its BLCA-4 expression as seen by PCR, Western blot, and its increased proliferation rate. Approximately 38,500 genes were screened using Affymetrix U133 Plus 2.0 human genome chips. To choose genes of interest for further analysis, a cutoff of 5-fold increase or decrease was chosen. It is acknowledged that this artificially defined cutoff will miss important changes of lower magnitude, but this point was chosen to examine gross changes in expression (Tables 1 and 2). Clustering analysis was done and a heat map of the genes was generated. A sample of the heat mapping from the altered genes is represented in Fig. 2.
blot analyses of selected proteins were done on whole cell lysates for further confirmation of the microarray data. The expression of IL-8, thrombomodulin, and IL-\(\alpha\) in whole cell lysates was examined by immunoblots. Expression of all three of these proteins correlated with the microarray data, as they were all overexpressed in the transfected clones compared with vector only controls (Fig. 3). Whereas the increase in IL8 may not be visibly obvious, densitometry indicates that the level of IL8 in clone 21 is increased over the vector only cells.

### Discussion

Despite much investigation, there remains little understanding of the basic biology of bladder cancer. Our lab has successfully identified six nuclear matrix proteins specifically expressed in bladder cancer and has developed urine-based immunoassays using antibodies raised to two of these proteins. Whereas we have successfully developed immunoassays for two of these proteins and have begun to elucidate the functional aspects of BLCA-4, we still have much to learn about the role of this protein in bladder cancer progression. The cDNA of BLCA-4 reveals that it seems a novel member of the ETS transcription factor family. It shares close homology with the ELK-3 member of this family and therefore may be a regulator of transcription. Insertion of the BLCA-4 gene into the T24 cell line, a transitional cell carcinoma line, has shown growth advantage for these cells (4).

It is recognized that the nuclear matrix plays a role in many functions that are implicated in cancer progression. The purpose of the studies done here was to further understand the role of the novel nuclear matrix protein, BLCA-4, in bladder cancer by altering expression levels of this gene in a cell model and examining the effects of this overexpression. We have successfully transfected the

### Table 1. Genes that are up-regulated in cells expressing BLCA-4 compared with vector only controls

<table>
<thead>
<tr>
<th>Vector only signal</th>
<th>Clone 21 only signal</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>201018_s_at</td>
<td>29</td>
<td>71.3</td>
</tr>
<tr>
<td>204614_at</td>
<td>23.3</td>
<td>60.7</td>
</tr>
<tr>
<td>204351_at</td>
<td>43.4</td>
<td>32.5</td>
</tr>
<tr>
<td>210544_s_at</td>
<td>47.8</td>
<td>25.5</td>
</tr>
<tr>
<td>206807_s_at</td>
<td>16.8</td>
<td>23.2</td>
</tr>
<tr>
<td>203887_at</td>
<td>22.7</td>
<td>20.5</td>
</tr>
<tr>
<td>204470_at</td>
<td>49</td>
<td>17.9</td>
</tr>
<tr>
<td>203888_at</td>
<td>13.9</td>
<td>13.0</td>
</tr>
<tr>
<td>202859_x_at</td>
<td>271.4</td>
<td>12.4</td>
</tr>
<tr>
<td>211506_s_at</td>
<td>175.6</td>
<td>11.4</td>
</tr>
<tr>
<td>201110_s_at</td>
<td>64.4</td>
<td>9.8</td>
</tr>
<tr>
<td>217999_s_at</td>
<td>18.9</td>
<td>9.8</td>
</tr>
<tr>
<td>210512_s_at</td>
<td>90.1</td>
<td>8.1</td>
</tr>
<tr>
<td>206864_s_at</td>
<td>9.1</td>
<td>8.0</td>
</tr>
<tr>
<td>205644_s_at</td>
<td>154.1</td>
<td>7.9</td>
</tr>
<tr>
<td>201109_s_at</td>
<td>118.6</td>
<td>7.7</td>
</tr>
<tr>
<td>205014_at</td>
<td>32.9</td>
<td>7.0</td>
</tr>
<tr>
<td>214300_s_at</td>
<td>14.5</td>
<td>6.6</td>
</tr>
</tbody>
</table>

**Description**

- IL-1\(\alpha\)
- Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2
- S100 calcium-binding protein P
- Aldehyde dehydrogenase 10 (fatty aldehyde dehydrogenase)
- Serum amyloid A2
- Thrombomodulin
- GRO1 oncogene (melanoma growth-stimulating activity, \(\alpha\))
- Thrombomodulin
- IL-8
- IL-8 COOH-terminal variant
- Thrombospondin 1
- Pleckstrin homology-like domain, family A, member 1
- Vascular endothelial growth factor
- Harakiri, BCL2-interacting protein (contains only BH3 domain)
- Tumor necrosis factor \(\alpha\)-induced protein 3
- Thrombospondin 1
- Heparin-binding growth factor binding protein
- Topoisomerase (DNA) III \(\alpha\)

### Table 2. Genes that are down-regulated in cells expressing BLCA-4 compared with vector only controls

<table>
<thead>
<tr>
<th>Vector only signal</th>
<th>Clone 21 only signal</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>201009_s_at</td>
<td>641.3</td>
<td>12.5</td>
</tr>
<tr>
<td>206662_at</td>
<td>197.5</td>
<td>11.8</td>
</tr>
<tr>
<td>210109_s_at</td>
<td>757.9</td>
<td>10.5</td>
</tr>
<tr>
<td>214599_s_at</td>
<td>311.7</td>
<td>10.5</td>
</tr>
<tr>
<td>202363_at</td>
<td>1,298</td>
<td>9.7</td>
</tr>
<tr>
<td>205440_at</td>
<td>369.1</td>
<td>7.9</td>
</tr>
<tr>
<td>201008_s_at</td>
<td>774.7</td>
<td>7.2</td>
</tr>
<tr>
<td>207826_s_at</td>
<td>948.1</td>
<td>6.5</td>
</tr>
<tr>
<td>219274_at</td>
<td>132.8</td>
<td>6.0</td>
</tr>
<tr>
<td>211420_at</td>
<td>2,733</td>
<td>5.4</td>
</tr>
<tr>
<td>207822_s_at</td>
<td>39.6</td>
<td>5.1</td>
</tr>
<tr>
<td>209122_at</td>
<td>1,826.5</td>
<td>4.9</td>
</tr>
<tr>
<td>207935_s_at</td>
<td>142.4</td>
<td>4.9</td>
</tr>
</tbody>
</table>

**Description**

- Up-regulated by 1,25-dihydroxyvitamin D-3
- Glutaredoxin (thioltransferase)
- Up-regulated by 1,25-dihydroxyvitamin D-3
- Involucrin
- Up-regulated by 1,25-dihydroxyvitamin D-3
- Testican-1 mRNA
- Cadherin 2, type 1, N-cadherin (neuronal)
- Inhibitor of DNA binding 3, dominant-negative helix-loop-helix protein
- Transmembrane 4 superfamily member (tetraspan NET-2)
- Vimentin
- 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
- Keratin 13
gene that encodes for BLCA-4 into a “normal” immortalized nontumorigenic cell line that does not express BLCA-4. The transfected clones had a significantly higher growth rate than the control cells so introduction of BLCA-4 therefore confers a growth advantage for cells.

Analysis of microarray data was done to further explore some of the genetic changes that may be occurring as a result of BLCA-4 expression. The results show a large number of genes that are either up-regulated or down-regulated in the cells expressing BLCA-4 in comparison with the vector only controls. Because of the considerable number of genes that were altered in the BLCA-4 expressing clones, a cutoff of 3-fold increase or decrease was used to filter out genes for further analysis. Many genes that are altered as a result of BLCA-4 expression have implications in cancer progression or as markers of disease. Three genes that are up-regulated in the BLCA-4-expressing clones, IL-1α, IL-8, and thrombomodulin were chosen for further analysis based on their level of expression and availability of commercial antibodies for use in the confirmation studies.

IL-1α is a major cytokine in the generation of an inflammatory response. There are two isoforms of this protein, IL-1α and IL-1β, although both forms seem to have very similar biological functions. Some effects of IL-1 in tumors have been reported. It seems that this protein can have both protumoral and antitumoral effects (10). IL-1 can have growth inhibitory effects on tumor cell lines, has exhibited antiangiogenic properties, and can also up-regulate host defenses and function as an immunostimulatory agent. However, it has also been reported that in other cases IL-1 may advance metastasis by promoting tumor cell-endothelial cell adhesion and can also increase expression of matrix-degrading enzymes such as urokinase-type plasminogen activator and stromelysins (10). Our microarray study showed a marked increase in IL-1α expression in cells transfected with the BLCA-4 gene. This increased expression was also verified at the protein level by immunoblots. There is not much literature available at this time exploring the expression of IL-1α in bladder cancer. However, the increase in IL-1α expression may be increasing the tumorigenic potential of the cells expressing BLCA-4.

![Figure 2](image1.png)

Figure 2. Examples of heat map generated following clustering of genes. A, genes up-regulated in clone 21 compared with vector only clone. B, genes down-regulated in clone 21 compared with vector only clone.

![Figure 3](image2.png)

Figure 3. Western blot analysis of IL1-α, IL-8, and thrombomodulin. Cells transfected with and overexpressing BLCA-4 show increased expression of IL1-α, IL-8, and thrombomodulin. Clone 21 expresses the highest level of BLCA-4 and also expresses the highest amounts of IL1-α, IL-8, and thrombomodulin.
IL-8, first identified as a leukocyte chemoattractant, is known to induce angiogenesis and is expressed by various tumors, including transitional cell carcinomas of the bladder. Expression of IL-8 also correlates with the metastatic potential of tumors (11). It has been discovered that levels of IL-8 are elevated in the urine from individuals with transitional cell carcinoma of the bladder (12). Additionally, human anti-IL-8 antibodies have shown the ability to inhibit tumor growth in vivo (13). Our microarray analysis showed a 12-fold increase in the expression of IL-8 in the clone overexpressing BLCA-4, showing the potential role of BLCA-4 in increasing tumorigenicity of bladder cancer.

An additional gene of interest that is up-regulated in the transfected clone is thrombomodulin. Thrombomodulin is a transmembrane glycoprotein that is involved in regulating intravascular coagulation. The expression of thrombomodulin in bladder cancer has not been studied extensively. However, it has been shown that thrombomodulin can be a sensitive but nonspecific marker of urothelial carcinoma (14). Plasma levels of thrombomodulin have been reported to increase with progression of malignant disease of various cancers (15). There is a great increase in thrombomodulin protein expression in the BLCA-4-expressing clone when compared with the vector only clone.

There are other interesting genes to note that are up-regulated in the BLCA-4-expressing cells that were not further confirmed at the protein level by immunoblots and may have a potential effect in bladder cancer progression. Vascular endothelial growth factor is an angiogenic agent that has been reported to be up-regulated in a multitude of tumors. Serum amyloid is a cytokine that is increased as a result of inflammation or injury and can also influence cell motility, adhesion, and proliferation and may be a potential serum marker of various diseases, including lung cancer (16). Genes found to be down-regulated in the BLCA-4-transfected cells were also analyzed. Vitamin D3 up-regulated protein was down-regulated in our comparison. It is found to be down-regulated in some tumor types and has been suggested to have tumor suppressor activity (17). There are a great number of genes that are altered by BLCA-4 expression that can be further explored as components of other studies.

Some of the potential pathways through which BLCA-4 aids in the pathobiology of bladder cancer are outlined in Fig. 4. BLCA-4 overexpression has been shown to up-regulate IL1-α. IL1-α in turn can increase the expression of matrix-degrading enzymes as well as promote tumor cell endothelial cell adhesion. As a result of overexpression of IL1-α could ultimately lead to enhanced proliferation and invasion of bladder tumor cells. BLCA-4 overexpression also results in increased expression of thrombomodulin, an anticoagulant. Tumor vasculature is leaky and can be prone to being plugged with blood clots thus blocking blood flow to the tumor. Anticoagulants aid in maintaining the blood flow necessary for tumor cell survival. Additionally, overexpression of BLCA-4 leads to increased expression of IL8. Research indicates that IL8 increases angiogenesis therefore providing necessary blood flow to tumors.

The studies outlined in this article provide more insight into the nuclear matrix protein, BLCA-4, which has already shown great promise as a marker of bladder cancer. We have shown that overexpression of BLCA-4 can successfully be achieved by stable transfection into bladder cells and confers a growth advantage in this cell model. We have also begun to elucidate some of the possible roles that BLCA-4 may play in the pathobiology of bladder cancer by doing microarrays. As a result of this analysis, a number of genes that are involved in cancer progression have been found to be altered by BLCA-4 and may be targets for future research. It is interesting to note that clone 21 expresses the highest level of BLCA-4 at both the message and protein levels and also shows the
most noticeable increase in IL-1α, IL-8, and thrombomodulin expression. It seems that expression of BLCA-4 not only increases the growth rate in cells but causes cells to express a more tumorigenic phenotype. Additional studies will be done in animal models to examine the tumorigenic potential of BLCA-4.

Acknowledgments


Grant support: NIH grant RO1 CA82522.

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.

References

Retraction: Mechanistic Analysis of the Role of BLCA-4 in Bladder Cancer Pathobiology

The authors wish to retract the article titled "Mechanistic Analysis of the Role of BLCA-4 in Bladder Cancer Pathobiology," which was published in the August 15, 2005 issue of Cancer Research (1).

The studies performed in the article used a cDNA sequence that at the time of publication the authors believed to encode BLCA-4. The authors feel that although this is still likely the case, there currently exists some uncertainty whether the cDNA sequence indeed encoded this protein. The authors believe that the work performed in the article is fully supported, but that the conclusions reached may differ based upon this potential; therefore, the authors felt that retraction of the article was warranted, and sincerely apologize for any inconvenience this might have caused.

Two of the three authors agreed to this Retraction. Attempts on the part of the authors to contact the second author, Thu-Suong Van Le, were unsuccessful.

Julie M. Myers-Irvin and Robert H. Getzenberg
Departments of Urology, Pathology, and Pharmacology, University of Pittsburgh and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Reference


Published online February 3, 2014.
doi: 10.1158/0008-5472.CAN-13-3721
©2014 American Association for Cancer Research.
Mechanistic Analysis of the Role of BLCA-4 in Bladder Cancer Pathobiology

Julie M. Myers-Irvin, Thu-Suong Van Le and Robert H. Getzenberg