

# Clinical Cancer Research



## Functional Characterization of the Bladder Cancer Marker, BLCA-4

Thu-Suong Van Le, Julie Myers, Badrinath R. Konety, et al.

*Clin Cancer Res* 2004;10:1384-1391.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/10/4/1384>

**Cited Articles** This article cites by 11 articles, 5 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/10/4/1384.full.html#ref-list-1>

**Citing articles** This article has been cited by 5 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/10/4/1384.full.html#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, contact the AACR Publications Department at [permissions@aacr.org](mailto:permissions@aacr.org).

# Functional Characterization of the Bladder Cancer Marker, BLCA-4

Thu-Suong Van Le,<sup>1</sup> Julie Myers,<sup>1</sup>  
Badrinath R. Konety,<sup>2</sup> Timothy Barder,<sup>3</sup> and  
Robert H. Getzenberg<sup>1</sup>

<sup>1</sup>Department of Urology, University of Pittsburgh and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania; <sup>2</sup>Department of Urology, University of Iowa, Iowa City, Iowa; and <sup>3</sup>Eprogen Inc., Darien, Illinois

## ABSTRACT

**Introduction:** Bladder cancer is a common disease of the genitourinary tract for which the development of a noninvasive detection technique would have a significant impact on disease management. One of our previously identified bladder cancer-specific proteins, BLCA-4, appears to be associated with a “field effect” of the disease, and in clinical trials is able to separate individuals with bladder cancer from those without the disease with high sensitivity and specificity. The potential clinical utility of this marker has led to the analysis of its function in bladder cancer pathobiology.

**Experimental Design:** To additionally analyze the specificity of this marker, the expression in the urine of a variety of benign urologic conditions was analyzed. After cloning the gene encoding BLCA-4, functional aspects of the protein were analyzed by overexpressing it in cell systems, as well as its interaction with other transcription factors and in gel mobility shift assays. Finally, to determine the timing of expression in relation to the observance of bladder cancer, an animal model of the disease was examined.

**Results:** Expression of BLCA-4, the cDNA of which reveals that it is a novel member of the ETS transcription factor family, is not found in benign urologic conditions. Overexpression leads to increased growth rates of cells, and the protein interacts with other transcription factors. *In vivo* studies reveal that BLCA-4 expression occurs significantly before the observance of grossly visible tumors in an animal model of the disease.

**Conclusions:** BLCA-4 is a bladder cancer marker that is highly specific and occurs early in the development of the disease. It appears to be a transcription factor that may play

a role in the regulation of the gene expression in bladder cancer. BLCA-4 is a marker with significant clinical utility that may have an active role in the disease.

## INTRODUCTION

According to the American Cancer Society (1),<sup>3</sup> >60,240 new cases of bladder cancer will be diagnosed in the United States and 12,710 people will die of the disease. Approximately, 90% of tumors of the urinary bladder in the United States are of transitional cell type, 7% are squamous, 2% are glandular, and 1% are undifferentiated (2). The bladder, in particular, serves as a reservoir for concentrated excretory products, and as such, makes it particularly vulnerable to environmental carcinogens. Exposure to certain chemicals (aromatic amines) and cigarette smoking are known risk factors. Moreover, epidemiologists estimate that 10–50% of bladder cancers may result from environmental exposures (3). The most common indication of bladder cancer is blood in the urine known as hematuria, occurring at initial presentation in ~80% of patients (4). In bladder cancer, hematuria may appear suddenly with no apparent cause, with typically no pain on presentation. Other irritating symptoms (also common in patients with benign prostatic hyperplasia) may include urinary urgency and frequency, painful urination, and abdominal pain. However, it is also important to note that hematuria typically presents itself due to other diseases such as bladder infections or kidney stones. It does not necessarily indicate bladder cancer. In fact, most people presenting with hematuria (>90%) will not have the disease.

The 5-year survival rate for superficial transitional cell carcinoma of the bladder is 94%, whereas disease that has spread regionally or distantly results in survival rates of 48% and 6%, respectively.<sup>3</sup> Therefore, early diagnosis is crucial in the effective treatment for patients who have bladder cancer. Cystoscopy remains the gold standard for the detection of bladder cancer. In conjunction with cystoscopy is urine cytopathology, which allows for the identification of precancerous and cancerous cells. A simple and noninvasive test to screen for these bladder cancer patients could increase significantly the prognosis for recovery in these patients.

Our previous studies (5, 6) describe the isolation of six proteins (BLCA-1 to -6) found to be uniquely expressed in the tumor tissue of patients with bladder cancer. BLCA-4, one of the most abundant of these proteins, was isolated by excising gel spots from negatively stained two-dimensional gels. The gel spots were then concentrated to obtain protein sequences and synthesized for antibody production. The first resulting peptide sequence was EISQLNAG, with a 75% homology with a number of nonvertebrate proteins including the epidermin biosyn-

Received 3/24/03; revised 10/23/03; accepted 11/10/03.

**Grant support:** NIH 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.

**Requests for reprints:** Robert H. Getzenberg, Department of Urology Research Laboratories, University of Pittsburgh, Suite G40, Shadyside Medical Center, 5200 Centre Avenue, Pittsburgh, PA 15232. Phone: (412) 623-3903; Fax: (412) 623-3904; E-mail: getzenbergrh@msx.upmc.edu.

<sup>3</sup> Internet address: <http://www.cancer.org>.

thesis protein, whereas the second peptide of BLCA-4 was VYEDIMQK, with a 75% homology with the ERECTA receptor protein kinase according to the BLAST database.<sup>4</sup> In summary, it is not apparent that either of the two peptide sequences for BLCA-4 shared significant similarity to any known human proteins.

To investigate whether we could detect the BLCA-4 protein in the urine of individuals with bladder cancer, 106 patients were enrolled in a study, whereby their urine samples were collected and tested using a standard ELISA (7) using a polyclonal antibody raised to a fragment of the BLCA-4 antigen. More specifically, the collection of urine samples included 55 bladder cancer patients and 51 normal volunteers, and an ELISA cutoff of 13 A/ $\mu$ g was established with the first set of the three tumor and normal volunteer samples, and applied prospectively. Anything below the 13 A/ $\mu$ g value was considered normal, whereas anything above would be indicative of bladder cancer. The results revealed that the 51 normal volunteers had an average value of  $4.02 \pm 4.21$  A/ $\mu$ g, and the bladder cancer patient values were  $43.36 \pm 49.52$  A/ $\mu$ g of protein. The levels were clearly significantly higher in patients with bladder cancer ( $P = 2.2 \times 10^{-18}$ ). The cutoff value yielded a sensitivity of 96.4% (53 of 55) with confidence interval = 87.5–99.6% and a specificity of 100% (51 of 51) with confidence interval = 93–100%. The BLCA-4 levels under the 13 A/ $\mu$ g cutoff, however, are not necessarily reflective of levels of the protein in normal individuals but may just indicate the background of the assay developed. A recent study using a sandwich assay to detect BLCA-4 in straight urine samples has been performed on a large population of individuals with bladder cancer along with those with benign urologic conditions, other cancer types, and normal controls. This study again demonstrated a specificity of 95% along with a sensitivity of 89%.<sup>5</sup> Despite the complexity and size of the population studies, BLCA-4 continues to be highly specific for bladder cancer.

The goal of the studies outlined in this article is to begin to define the functional aspects of BLCA-4. After the identification and isolation of the gene encoding this protein, it was necessary to analyze potential effects of the encoded protein by reintroducing it into various cell types and examine changes in the cells. Furthermore, the activity of the encoded protein under a variety of physiological conditions can also be examined. By reintroducing the BLCA-4 gene into a cell line that does not express or have low expression level of BLCA-4, we are able to study the effects on cellular growth as well as other functional aspects of the cells. Additionally, these methods allow large-scale production and purification of the protein product for biochemical characterization. Finally, as a potential marker of the field effect associated with bladder cancer, we examined the development of bladder cancer in concert with the expression of BLCA-4 using an animal model of the disease.

## MATERIALS AND METHODS

**Immunoblot Analysis.** Immunoblot analysis was performed according to the standard established protocols. Twenty  $\mu$ g of each extracted sample of nuclear matrix proteins suspended in PBS (0.137 M sodium chloride, 0.0027 M potassium chloride, and 0.0119 M phosphates) were loaded and separated by 12% SDS-PAGE along side a molecular weight standard (Amersham Life Sciences, Arlington Heights, IL). The proteins were then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated overnight at 4°C in blocking solution consisting of 5% nonfat dry milk, 1 $\times$  PBS, and 0.2% Tween 20. Membranes were washed the following day in 1 $\times$  PBS and 0.2% Tween 20 before and after the incubation of primary and secondary antibodies. The standard dilution used for the primary antibody (anti-BLCA-4) is 1:500, whereas the secondary antibody goat- $\alpha$ -rabbit conjugated to horseradish peroxidase (Amersham Life Sciences) is diluted to 1:5000. Both the primary and secondary antibodies were incubated in the blocking solution. The proteins were detected by the chemiluminescence reaction using the ECL immunoblot kit (Amersham Life Sciences).

**mRNA Isolation and cDNA Synthesis.** Two peptide sequences corresponding to a distinct region of BLCA-4 were identified and used in raising antibodies against these proteins. The characterization of BLCA-4 involved constructing cDNA libraries using mRNA isolated from both the human bladder cancer cell line T24 (American Type Culture Collection, Manassas, VA) and human bladder donor tissue obtained from the University of Pittsburgh tissue banking facility. It was necessary to begin with  $1 \times 10^7$  cells and at least 100 mg of tissue for mRNA isolation. The T24 cells were trypsinized, washed, and resuspended in RNA extraction buffer. The donor bladder tissues were minced by a tissue homogenizer in the same extraction buffer. Polyadenylated RNA was then isolated using the Quick Prep Micro mRNA Purification Kit (Pharmacia Biotech, Piscataway, NJ). From the purified polyadenylated RNA, the first strand of cDNA was synthesized using the SuperScript Preamplification System (Life Technologies, Inc., Gaithersburg, MD). This system was involved in priming first-strand cDNA synthesis with random hexamers in preparation for Hot Start reverse transcription-PCR. The 3' end of the cDNA for BLCA-4 was additionally extended by using the 3' Rapid Amplification of cDNA ends kit (Invitrogen).

**Cloning of BLCA-4.** All of the possible primer combinations were synthesized from the degenerate oligonucleotides sequenced previously for BLCA-4. The degenerate primers were based on the encoded peptide sequences, EISQLNAG and VYEDIMQK. The resulting PCR products were purified using the DNA Purification System (Promega, Madison, WI). After amplification and purification of the DNA, the reverse transcription-PCR products were blunt-ended with T4 DNA polymerase and kinased with T4 polynucleotide kinase, each followed by a phenol extraction. The cDNA was then cloned into the pCRII vector (Invitrogen, Carlsbad, CA) and subsequently subcloned into the pcDNA3.1/*lacZ* vector (Invitrogen) using T4 DNA ligase and transfected using a standard electroporation protocol in TOP10 competent cells (Invitrogen). Furthermore, the plasmid DNA was isolated from bacterial cells by a variation of

<sup>4</sup> Internet address: <http://www.ncbi.nlm.nih.gov/blast>.

<sup>5</sup> T-S. Van Le, R. Miller, T. Barder, M. Babjuk, D. M. Potter, and R. H. Getzenberg. A highly specific urine-based marker of bladder cancer, submitted for publication.

alkaline lysis method and concentrated by ethanol precipitations. A total of 0.8  $\mu\text{g}/\mu\text{l}$  of DNA was sent to a cycle sequencing facility (University of Chicago) to confirm insertion and proper orientation of the gene encoding for BLCA-4.

**Antigen Detection.** Urine samples were obtained for analysis from patients with various urologic conditions (bladder cancer, hematuria, infection, and other types of cancer). Urines from individuals with known diagnoses were sent to our facility as part of a multicenter clinical trial study. The samples were processed, bar coded for easy identification, and stored in a  $-20^{\circ}\text{C}$  freezer. One-dimensional and two-dimensional immunoblots were run by standard methods and techniques. Twenty  $\mu\text{g}$  of protein were loaded into each lane in a blinded fashion, and each membrane was probed with the anti-BLCA-4 antibody at a 1:500 dilution.

#### Early Marker of Bladder Cancer in Animal Studies.

Because it would be virtually impossible to measure the course of BLCA-4 expression in humans, an animal model has been established to study the progression of bladder cancer. Developed by Hicks and Wakefield (8, 9) and further modified by Steinberg *et al.* (10), the animal model involved delivering the carcinogen *N*-methyl-*N*-nitrosourea (MNU) directly into the rat bladders to induce the development of bladder cancer. Tumors do not occur until about week 30, and most of these are transitional cell carcinomas. This model has been extensively studied, karyotyped, and successfully used for analyzing the effect of other intravesical therapeutic agents (11). Briefly, 57 female F344 Fisher rats underwent intravesical instillation of the MNU on weeks 0, 2, 4, and 6. The instillations were performed after catheterizing the bladders with a 3F tomcat catheter under halothane anesthesia. After completion of four doses of MNU, the animals did not receive additional treatments. Two animals were sacrificed on weeks 8, 16, 20, 24, 26, 30, and 34. The bladders of the sacrificed animals were harvested and preserved in 8 mM PBS with 1.2 mM phenylmethylsulfonyl fluoride. Urine samples were collected from weeks 2 and 4 of both the normal controls and MNU animals. Nuclear matrix proteins were extracted from pooled tissue samples at the same time points and additionally analyzed on immunoblots.

**Transfection of BLCA-4.** The BLCA-4 cDNA was introduced into T24 cells (American Type Culture Collection), a human bladder tumor cell line. The transfection method used was the Lipofectamine Plus kit (Invitrogen). For each well of a six-well plate, a various amount of DNA (0, 1, 2, 4, 5, and 6  $\mu\text{g}$ ) was transfected into T24 cells ( $4 \times 10^4$  cells/well). For a stable transfection, selection began 48 h after transfection using G418. Each clone was isolated using clonal discs and transferred to a 24-well plate. Once confluent, the transfected cells were gradually transferred to a six-well, then T25  $\text{cm}^2$  and subsequently T75  $\text{cm}^2$  flasks to isolate cell lysates. Cell lysates were isolated from the clones and additionally analyzed in a number of functional assays. Immunoblot analysis was performed as described in Konety *et al.* (7).

*In vitro* studies of the transfected clones were evaluated in growth assays. Ten thousand cells for both transfected and nontransfected samples were seeded into T175- $\text{cm}^2$  flasks (4 flask each per sample). Each cell line was harvested and counted with a Coulter counter at days 6, 8, 10, and 13.

**Transcription Factor-Transcription Factor Interactions.** Using the NE-PER kit (Pierce, Rockford, IL), nuclear extracts from sense clones, including a vector-only control, were chosen based on the high levels of BLCA-4 expression by immunoblots analysis. The nuclear extracts were used to analyze the interaction of BLCA-4 with other multiple transcription factors in one experiment using the TranSignal TF-TF Interaction Arrays I and II (Panomics, Redwood City, CA). The three nuclear extracts selected include a nontransfected control, transfected sense clone 83, and transfected sense vector only clone 96. Together both Arrays I and II can potentially examine 105 unique transcription factors.

**Electromobility Shift Assays.** Electromobility shift assays were performed to analyze the interaction between BLCA-4 and known consensus DNA binding sequences. The recombinant protein was isolated by the PinPoint Xa Purification System (Promega). Briefly, the DNA oligo, *e.g.*, ETS oligo, was labeled with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (ICN, Aurora, OH) with T4 Kinase at  $37^{\circ}\text{C}$  for 1 h. The labeled probe was mixed with the binding buffer, poly(deoxyinosinic-deoxycytidylic acid) and BLCA-4 recombinant protein. The positive control used for the assay was the cell lysate from LNCaP cells. The sample mixture was incubated for 30 min at room temperature and loaded onto a 5% nondenaturing polyacrylamide gel. The gels were run at 100 V for  $\sim 50$  min and exposed to film.

## RESULTS

As described above, we have developed previously an immunoassay, which can detect BLCA-4, and which separates individuals with bladder cancer and separates them from those without the disease. To additionally characterize the expression of this antigen in tissue and urine samples, we performed both one-dimensional and two-dimensional immunoblot analyses of bladder tissues and urine samples from individuals with and without bladder cancer. Fig. 1 shows the results for the two-dimensional immunoblot analysis detailing the BLCA-4 levels of expression in both tissue (Fig. 1A) and urine samples (Fig. 1B) of patients with and without bladder cancer. Fig. 2 additionally illustrates the specificity of the BLCA-4 antigen expression for bladder cancer detection using urine samples collected from individuals having diagnoses of various urologic conditions, *e.g.*, bladder cancer, normal, hematuria, and so forth. The one-dimensional immunoblots in Fig. 2 clearly indicate the presence of the urinary protein BLCA-4 in patients with bladder cancer compared with the normal individuals and those with other urologic conditions. The individuals with bladder cancer from whom the urine samples were obtained had grade I (3), grade II (2), or grade II/III lesions (1). BLCA-4 was detected in the urine of all of the individuals with bladder cancer tested. BLCA-4 was absent in all of the other sample types tested except for possible small amount of staining in a single BPH sample, although this lane of the gel was not optimal for interpretation.

The anti-BLCA-4 antibody used in these immunoblots experiments is, therefore, able to differentiate between the normal and tumor types, in both tissue and in urine samples. The fact that this antibody is able to identify the BLCA-4 antigen in normal tissues from every patient with bladder cancer suggests

that this antibody may be able to detect very early lesions of bladder cancer, even before any morphological alterations are visible. This is additionally supported by examining the time course of BLCA-4 expression in the development of bladder cancer using the MNU animal model of the disease. The results for the one-dimensional immunoblots using the same anti-BLCA-4 antibody show that BLCA-4 is expressed in the bladders (Fig. 3) of rats at weeks 8–34, as well as in the urine (Fig. 4) at weeks 2, 4, and 37 after MNU exposure. These results indicate that BLCA-4 antigen is absent in the normal rat bladder

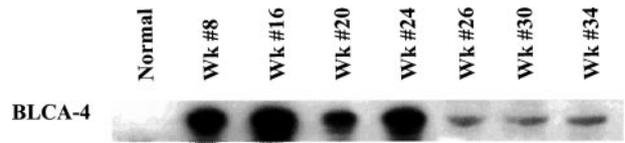


Fig. 3 Immunoblot analyses of BLCA-4 expression in the tissues from the rat *N*-methyl-*N*-nitrosourea (MNU) model. Detection of the BLCA-4 protein in the bladders of rats at various time points that have been induced to form bladder tumors by intravesicular administration of MNU.

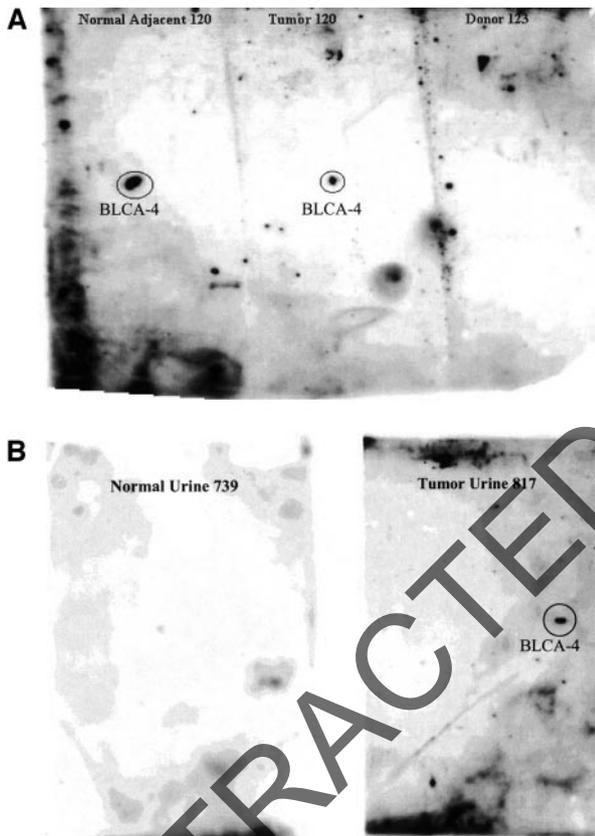


Fig. 1 *A*, two-dimensional immunoblots of tissue samples. Detection of the BLCA-4 protein in normal, tumor, and donor tissues from patients with bladder cancer. *B*, two-dimensional immunoblots of urine samples. Detection of the BLCA-4 protein in the urine of patients with and without bladder cancer.



Fig. 4 Immunoblot analysis of BLCA-4 expression in the urine from the rat *N*-methyl-*N*-nitrosourea (MNU) model. Detection of the BLCA-4 protein in the urine of rats at weeks 2 and 4 for both the normal and MNU-induced animals.

and urine but present in all of the bladders obtained from rats at each time point examined after MNU administration. The normal rat bladder shown is representative of the normal rat bladders of various ages and control treatments all of which were negative for BLCA-4 expression. Moreover, the preliminary data indicate that BLCA-4 antigen expression appears before the detection of grossly visible tumors in these animals. Therefore, BLCA-4 expression before the observance of any grossly visible tumors in this model strongly suggests that it might be an early onset disease marker in the development of bladder cancer. The observations in the MNU animal model correlate well with the expression patterns observed in normal and tumor human bladders.

The gene encoding BLCA-4 was cloned into the pcDNA 3.1 expression vector, and the resulting sequence was entered into the NIH sequence database (GenBank). Sequence homology information reveals that BLCA-4 has similarity to a previously identified member of the ETS transcription factor family with closest similarity to the *ELK-3* gene. Common regions include a nuclear localization sequence and helix-loop-helix region (Refs. 12, 13; Fig. 5).

The BLCA-4/pcDNA3.1 plasmid DNA was stably transfected in bladder epithelial T24 cells. Selection with the appropriate antibiotics began 48 h after transfection, and clones were selected 6 days later. A total of 92 transfected sense BLCA-4 clones were picked and compared with 4 transfected vector only clones. Forty-eight h after transfection, cells expressing the *lacZ* gene product,  $\beta$ -galactosidase, were stained using the  $\beta$ -Gal Staining kit (Invitrogen), as shown in Fig. 6, *A* and *B*.

Stably transfected cells were examined for BLCA-4 ex-

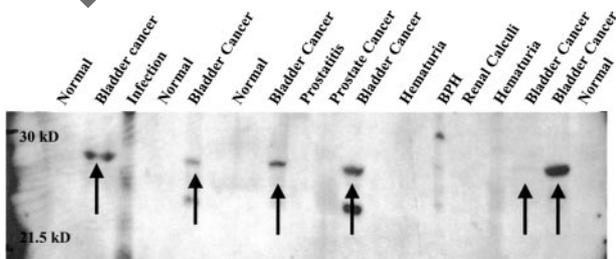


Fig. 2 One-dimensional immunoblot of urine samples. Detection of the BLCA-4 protein in the urine of patients with various diagnoses.

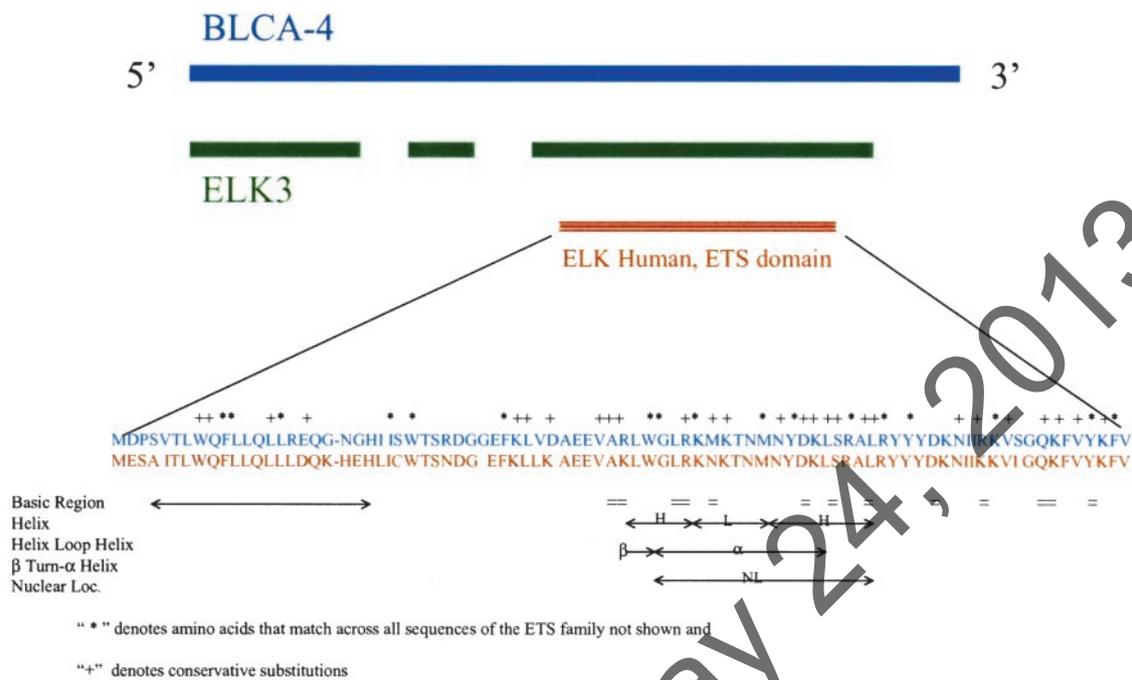


Fig. 5 Sequence homology of BLCA-4 with the ETS transcription factor family. The BLCA-4 protein has closest similarity with the *ELK3* gene.

pression using an anti-BLCA-4 antibody. The three transfected clones were selected that exhibited the highest BLCA-4 expression levels, and two vector-only controls were used for the remainder of our studies. To investigate a potential role in growth control for BLCA-4, we conducted growth assays on each of the transfected cell populations. The results reveal that there is a significant increase in cell growth in BLCA-4 overexpressing cells compared with the control vector only (Fig. 7). Overexpression of these lines provides a 60% growth advantage over the control transfectants (Fig. 8A). Additional analysis of growth regulation by BLCA-4 was conducted by evaluating expression of proliferating cell nuclear antigen in total cell lysates. The proliferating cell nuclear antigen expression patterns when corrected for actin levels reveal a 1.5–2.1-fold increase in expression over the vector-only control. Both cell growth and proliferating cell nuclear antigen assays reveal that BLCA-4 overexpression leads to a significant growth advantage (Fig. 8B).

To examine a potential role for BLCA-4 in regulating gene expression in bladder cancer, we studied its interaction with other known transcription factors (TFs). An estimated 2000 TFs are involved in any given human cell. The interaction of BLCA-4 with other TFs was examined by observing the interactions of cells expressing high levels of BLCA-4 compared with the control-transfected cells. The nuclear extracts of non-transfected, transfected BLCA-4, and control vector-transfected cells were compared. Fig. 9 identifies the interaction of unique transcription factors (Table 1) in the transfected clone compared with the nontransfected or to the vector-only control.

Furthermore, DNA-binding proteins have been studied because of their involvement in cellular processes such as replication and transcription. Many of the characterized nuclear

matrix proteins are known to bind DNA. Therefore, the interest in characterizing the DNA sequences responsible for the gene regulation has become increasingly important. This analysis has naturally led to the detection, isolation, and characterization of the proteins that bind to these regulatory sequence elements, by electromobility shift assays. Electromobility shift assays were performed using the ETS oligo (Santa Cruz Biotechnology, Santa Cruz, CA), which has demonstrated binding with a number of the transfected clones in comparison with the vector-only controls (data not shown).

## DISCUSSION

The early detection of bladder cancer is central to its effective therapy. Nuclear matrix proteins have been identified as fingerprints of bladder cancer separating it from the bladder of individuals without the disease. BLCA-4 has been shown to stain the entire bladder, including the tumor and normal adjacent areas of individuals that have bladder cancer. In addition, it can be detected in the urine of patients with bladder cancer allowing the separation of those with the disease from individuals without the disease with a sensitivity of 96.4% and a specificity of 100%. Studies in individuals with spinal cord injury and recent unpublished data demonstrate that BLCA-4 is not found in the urine of individuals with other benign conditions including cystitis and BPH, as well as being absent in the urine of individuals with other cancer types. In addition to continuing to follow the individuals with spinal cord injuries, a large-scale clinical trial is currently underway to validate these initial studies.

The nuclear matrix plays an active role in nuclear functions, which include DNA organization, the regulation of gene

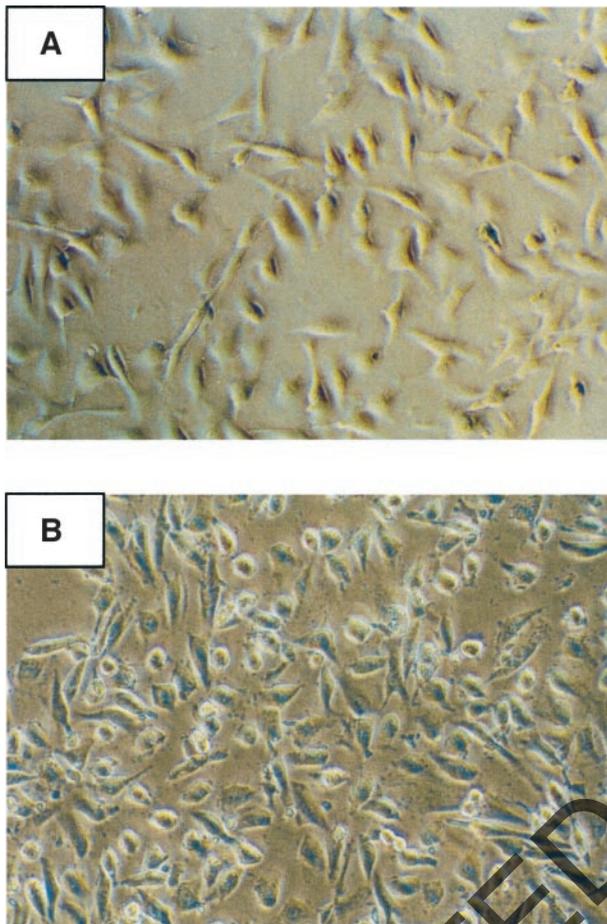


Fig. 6  $\beta$ -Galactosidase staining of T24 cells. Blue staining is evident in the transfected T24 cells (B) compared with the nontransfected cells (A).

expression, and replication. It is now well established that the nuclear matrix is altered in the cancer cell. Our group together with others has proposed that these alterations in nuclear structure may coincide with the hallmark of the cancer cell, changes in nuclear shape, along with fundamental losses in the fidelity of nuclear processes. The studies outlined in this article were designed to begin to elucidate the potential function of BLCA-4 in the process of bladder cancer. To determine what role BLCA-4 might have, if any, in bladder pathobiology, we cloned the gene that encodes the protein. Using the peptide sequences obtained from sequencing, the spots isolated from high-resolution two-dimensional electrophoresis sequence data were obtained for several peptides. These peptides were then used to construct degenerate PCR primers. These primers were then used to specifically amplify a portion of the cDNA library that we constructed from the T24 bladder cancer cell line and comparisons made with a library produced from the bladders of organ donors (normal controls). A resulting PCR product was identified and sequenced confirming that it contained the regions encoding the peptides. When sequenced, we determined that the gene did not contain the entire cDNA. The product was extended using 3' rapid amplification of cDNA ends. When the sequence was compared with the available databases, it was

revealed that BLCA-4 encodes for what appears to be a novel member of the ETS transcription factor family. It has the closest relationship to the member ELK-3 with several areas differentiating it. The identification of BLCA-4 as a potential transcriptional regulator reveals significant information regarding its potential function in bladder cancer. A number of ETS transcription factor family members have been shown to be altered in cancer states (14).

Although our sequence analysis revealed significant information regarding the potential of BLCA-4 to serve as a regulator of gene expression in bladder cancer, additional analysis was necessary to determine whether it indeed bound to specific DNA sequences as well as other transcriptional regulators with which it might interact. Gel-shift analysis using a consensus ETS DNA-binding domain reveals that BLCA-4 does indeed interact with this binding sequence. Additional studies are required to identify other DNA sequences to which BLCA-4 may associate. A transcriptional complex is composed of a number of factors, which interact together. By binding to specific DNA elements, each TF facilitates a different functional role in gene expression. Traditionally, TF-TF interactions have been studied by coimmunoprecipitation and super-gel shift. However, both methods are not conducive to mapping every known TF-TF interaction, because these methods are notoriously time consuming and inefficient. The TranSignal TF-TF Array I and II enables us to determine how a particular TF interacts with multiple other TFs in one experiment. To determine any potential interactions that might exist between BLCA-4 and other known transcriptional regulators, we performed analysis by TF-TF arraying. This array reveals the association of BLCA-4 with several known factors including, AP-1, AP-2, NFATC, NF-E1, and NF-E2. These interactions need to be additionally validated but serve as additional pieces to the puzzle of determining the function of BLCA-4.

Although the demonstrated associations are important pieces of information, the true function of BLCA-4 is determined by examining the effects of overexpression of the protein. Using a cell model, we were successful in transfecting in a construct, which contains BLCA-4 under the control of a constitutive promoter. Several clones were selected that produced relatively large amounts of the protein. In comparison to the vector-only controls, these cell lines had growth rates that were significantly higher. Therefore, BLCA-4 overexpression appears to result in a growth advantage for these cells. The vector-only controls have significantly lower growth rates than the wild-type cells. This is attributed to the effect of the relatively high concentrations of G418 in which all of the transfected clones were grown. We are currently in the process of determining the effects of BLCA-4 on the cell cycle. In addition, we are looking at the tumorigenicity of these lines in immunocompromised animals.

As described above, previous examination of BLCA-4 expression indicates that it is found throughout the bladder of individuals with bladder cancer. This indicates the potential of this protein to be involved in a field effect for the disease. A central question that remained was whether expression of BLCA-4 was indeed an early event in the development of bladder cancer. These studies cannot be performed in humans, because we do not know which individuals will develop bladder

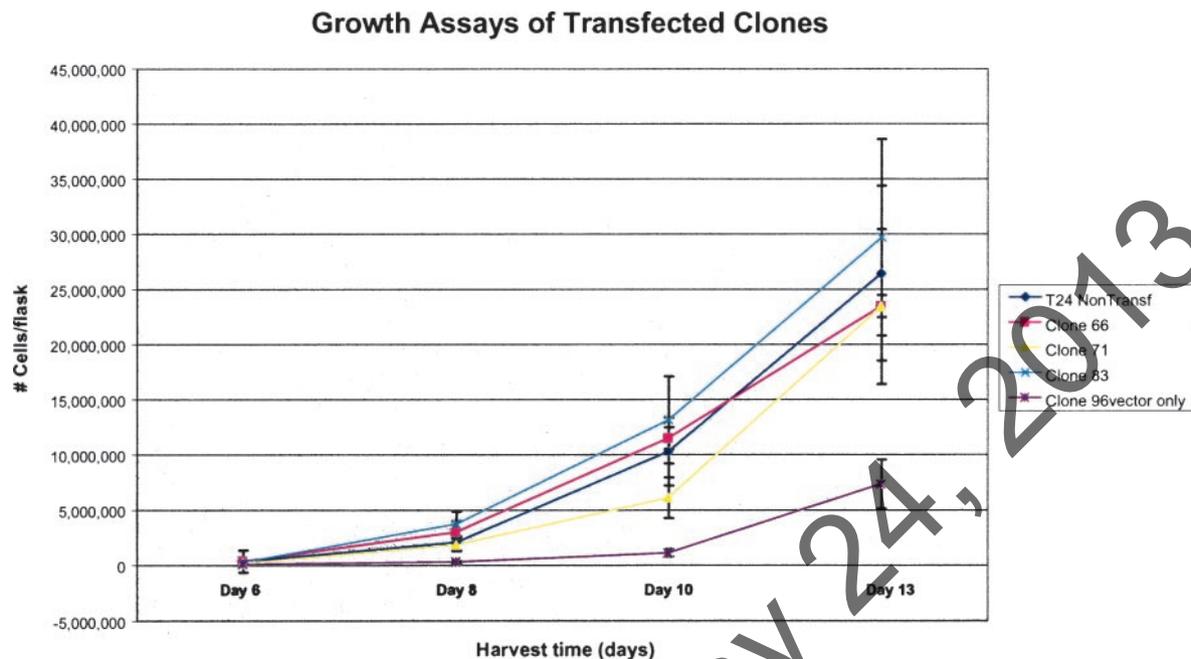


Fig. 7 Growth assays of transfected versus nontransfected T24 cells. Harvested and counted nontransfected T24 cells, transfected vector-only cells, and transfected BLCA-4 cells. Analyses were performed four times, and results are shown as a mean average percentage of 182% (bars,  $\pm 30\%$ ).

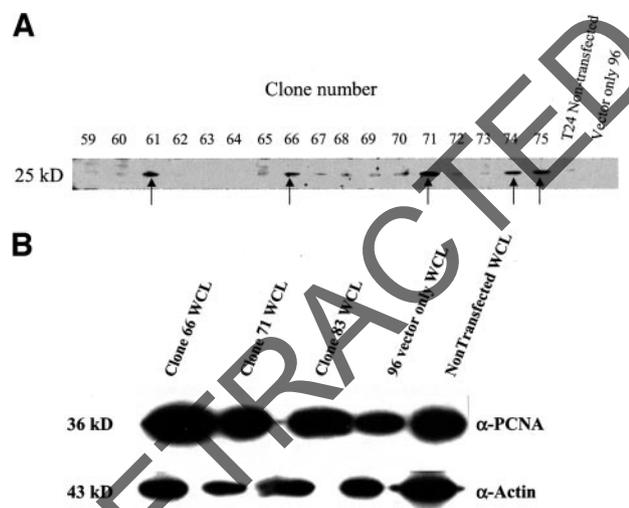


Fig. 8 A, immunoblot of transfected sense clones. Arrows are pointed at the selected clones with high expression levels of BLCA-4 as detected in immunoblots with the anti-BLCA-4 antibody. B, immunoblot of transfected BLCA-4 sense clones compared with the vector-only control. Proliferating cell nuclear antigen expression patterns when corrected for actin levels.

cancer before they get it. To decipher the timing of BLCA-4 expression, we studied an autochthonous model of bladder cancer. The MNU model of bladder cancer was selected because the animals reliably develop tumors by week 30, and the resulting tumors are transitional cell carcinomas in comparison with the squamous cell cancers that often result from some animal models. BLCA-4 is expressed at the earliest time point that we examined, 8 weeks after initiation of MNU administration and

as many as 22 weeks before the observance of tumors in this model. It is clear from these results that BLCA-4 is indeed a protein that is expressed quite early in the development of bladder cancer significantly before the development of gross tumors. One concern is the accuracy with which the antihuman

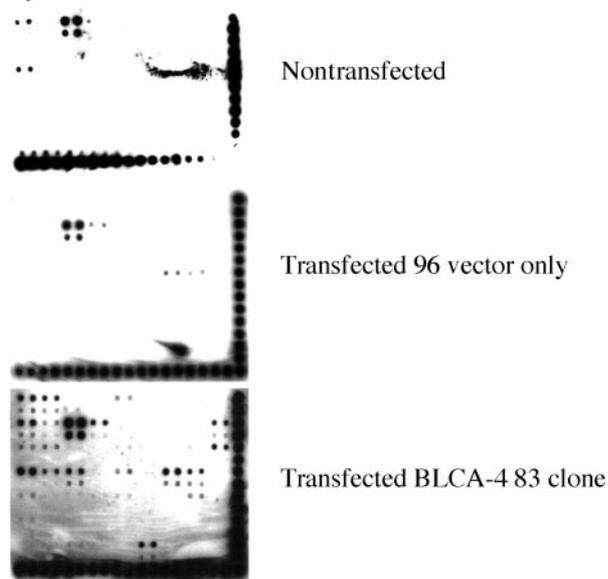


Fig. 9 TranSignal TF-TF Interaction Array I of nuclear extracts on T24 cells. Multiple transcription factors interacts with the transfected BLCA-4 clone #83 compared with both the nontransfected and transfected vector-only clone.

Table 1 Analysis of transcriptional regulators/association with BLCA-4

AP1 <sup>a</sup>	E2F-1 <sup>b</sup>	HNF-4	NFκB	RXR(DR-1)	Stat5
AP2	EGR	IRF-1	Oct—1	SIE	Stat5/Stat6
ARE	ERE	MEF-1	P53	Smad SBE	TFIID
Brn-3	Ets	MEF-2	Pax-5	Smad3/4 <sup>b</sup>	TR
C/EBP <sup>b</sup>	Ets-1/PEA3	Myc-Max	Pbx1	Sp1	TR(DR-4)
CBF	FAST-1	NF-1	Pit 1 <sup>b</sup>	SRE	USF-1
CDP	GAS/ISRE	NFATc <sup>a</sup>	PPAR	Stat1 p84/p91	VDR (DR-3)
c-Myb	GATA <sup>b</sup>	NF-E1 <sup>a</sup> (YY1)	PRE	Stat3	HSE
CREB <sup>b</sup>	GRE <sup>b</sup>	NF-E2 <sup>a</sup>	RAR(DR-5)	Stat4	MRE <sup>b</sup>

<sup>a</sup> High expression of transcription factor in clone #83.

<sup>b</sup> Expression of transcription factor in clone #83.

BLCA-4 antibody is able to identify rat proteins from the MNU-generated bladder cancers. We have additionally been able to identify BLCA-4 expression in homogenized protein extracts from pathologically confirmed rat tumor tissue obtained from other carcinogen-derived rat tumors (data not shown) by immunoblot analyses. Other urine-based tumor markers such as NMP22 have been identified in the urine of patients with a history of recurrent bladder cancer a few months before the appearance of the recurrent tumors (15). However, NMP22 tends to have a low specificity, and it is unclear if it is an early predictor of bladder cancer in individuals who have no prior history of bladder cancer. Because bladder cancer is acknowledged to be a field change phenomenon, presence of urinary NMP22 in individuals after an initial treatment for bladder cancer may be related to the presence of residual bladder tumor or cells affected by the field change effect. It may not indicate a true reoccurrence of tumor. The animal data from this study indicate that BLCA-4 may be expressed early in the process of carcinogenesis in bladders that have not had cancer previously.

The studies described in this article provide support for the concept that BLCA-4 is more than just a bladder tumor marker with high specificity. This nuclear protein is a novel member of the ETS transcription factor family that binds DNA and interacts with other known transcriptional regulators. Overexpression leads to increased growth rates in cell models. Finally, we are able to demonstrate that this protein is expressed early in the development of bladder cancer in an animal model and appears to be one of the earliest known changes associated with the disease. Additional studies are necessary to further delineate the mechanisms of BLCA-4 action and its role in bladder cancer pathobiology. Studies validating the clinical utility of the markers are currently underway.

## ACKNOWLEDGMENTS

We thank Dr. Uzma Shah for critical review of the article, as well as Drs. Mark Zeidel, Susan Meyers, and Anthony Kanai for providing tissue and urine samples for the MNU animal model.

## REFERENCES

1. American Cancer Society. Cancer Facts and Figures: 2003. Washington, DC: American Cancer Society, 2003.

- Mostafi, F. K., Davis, C. J., and Sesterhenn, I. A. Pathology of tumors of the urinary tract. *In*: D. G. Skinner and G. Lieskovsky (eds.), *Diagnosis and Management of Genitourinary Cancer*, pp. 83–117. Philadelphia: Saunders, 1988.
- Lower, G. M. Concepts in causality: chemically induced human bladder cancer. *Cancer (Phila)*, *49*: 1056, 1982.
- Murphy, W. M., and Soloway, M. S. Urothelial dysplasia. *J. Urol.*, *127*: 849–854, 1982.
- Getzenberg, R. H., Konety, B. R., Oeler, T. A., Quigley, M. M., Hakam, A., Becich, M. J., and Bahnson, R. R. Bladder cancer-associated nuclear matrix proteins. *Cancer Res.*, *56*: 1690–1694, 1996.
- Konety, B. R., Nguyen, T-S. T., Lavelle, J. P., Zeidel, M. L., and Getzenberg, R. H. Expression of BLCA-4, a nuclear matrix protein (NMP) marker in a carcinogen induced model of bladder cancer. *J. Urol.*, *163* (Suppl.): abstract 597, 2000.
- Konety, B. R., Nguyen, T-S, Dhir, R., Becich, M. J., Stadler, W. M., and Getzenberg, R. H. Detection of bladder cancer using a novel nuclear matrix protein, BLCA-4. *Clin. Cancer Res.*, *6*: 2618–2625, 2000.
- Hicks, R. M., and Wakefield, J. Rapid induction of bladder cancer in rats with *N*-methyl-*N*-nitrosourea I. *Histol. Chem. Biol. Interact.*, *5*: 139–152, 1972.
- Hicks, R. M., and Wakefield, J. Membrane changes during urothelial hyperplasia and neoplasia. *Cancer Res.*, *36*: 2502–2507, 1976.
- Steinberg, G. D., Brendler, C. B., Ichikawa, T., Squire, R. A., and Issacs, J. T. Characterization of an *N*-methyl-*N*-nitrosourea induced autochthonous rat bladder cancer model. *Cancer Res.*, *50*: 6668–6674, 1990.
- Raghavan, D., Debruyne, F., Herr, H., Jocham, D., Kakizoe, T., Okajime, E., Sandberg, A., and Tannock, I. Experimental models of bladder cancer: a critical review. *Prog. Clin. Biol. Res.*, *171*: 208, 1986.
- Wasylyk, B., Hanh, S. L., and Giovane, A. The Ets family of transcription factors, review. *Eur. J. Biochem.*, *211*: 7–18, 1993.
- Rao, V. N., Huebner, K., Isobe, M. Ar-Rushdi, A., Croce, D. M., and Reddy, E. S. P. Elk, tissue-specific ets-related genes on chromosomes X and 14 near translocation breakpoints. *Science (Wash. DC)*, *244*: 66–70, 1989.
- Mavrothalassitis, G., and Ghysdael, J. Proteins of the ETS family with transcriptional repressor activity, review. *Oncogene*, *19*: 6524–6532, 2000.
- Soloway, M. S., Briggman, J. V., Carpinto, G. A., Chodok, G. W., Church, P. A., Lamm, D. L., Lange, P., Messing, E., Pasciak, R. M., Reservitz, G. B., *et al.* Use of a new tumor marker, urinary NMP22, in the detection of occult or rapidly recurring transitional cell carcinoma of the urinary tract following surgical treatment. *J. Urol.*, *156*: 363–367, 1996.

## Retraction: Functional Characterization of the Bladder Cancer Marker, BLCA-4

The authors wish to retract the article entitled "Functional Characterization of the Bladder Cancer Marker, BLCA-4," which was published in the February 15, 2004, issue of *Clinical Cancer Research* (1).

The studies described in the article used a cDNA sequence that, at the time of publication, the authors believed to encode BLCA-4. Although this is still likely the case, there currently exists some uncertainty about whether the cDNA sequence indeed encoded this protein. The work described in the article is fully supported, but the conclusions reached may differ based on this potential. The authors apologize for any inconvenience.

**Thu-Suong Van Le**

**Julie Myers**

**Robert H. Getzenberg**

Department of Urology, University of Pittsburgh and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

**Badrinath R. Konety**

Department of Urology, University of Iowa, Iowa City, Iowa

**Timothy Barder**

Eprogen Inc., Darien, Illinois

### Reference

1. Van Le TS, Myers J, Konety BR, Barder T, Getzenberg RH. Functional characterization of the bladder cancer marker, BLCA-4. *Clin Cancer Res* 2004;10:1384-91.

Published OnlineFirst May 24, 2013.

doi: 10.1158/1078-0432.CCR-13-1145

©2013 American Association for Cancer Research.

# Clinical Cancer Research

## Functional Characterization of the Bladder Cancer Marker, BLCA-4

Thu-Suong Van Le, Julie Myers, Badrinath R. Konety, et al.

*Clin Cancer Res* 2004;10:1384-1391.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/10/4/1384>

**Cited articles** This article cites 12 articles, 5 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/10/4/1384.full#ref-list-1>

**Citing articles** This article has been cited by 8 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/10/4/1384.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://clincancerres.aacrjournals.org/content/10/4/1384>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.