Overexpression of Vimentin: Role in the Invasive Phenotype in an Androgen-independent Model of Prostate Cancer

Sadmeet Singh, Skanda Sadacharan, Scott Su, Arie Beldegrun, Sujata Persad, and Gurmit Singh

ABSTRACT

The androgen-sensitive LNCaP prostate cancer cell line is less invasive than hormone-insensitive lines. CL1, an aggressive, hormone-insensitive LNCaP subline derived by continuous passage in hormone-depleted medium, was compared with the parental cell line by cDNA microarray analysis. The gene coding for the intermediate filament protein vimentin was found to be highly up-regulated in the CL1 subline. This difference was confirmed by Northern and Western blots and visualized by immunofluorescence microscopy. To assess the contribution of vimentin to the invasive phenotype, LNCaP cells were stably transfected to overexpress vimentin, and the CL1 cells were transfected with vimentin antisense construct. The invasiveness of the transfected cells was tested using an in vitro invasion assay. We were able to demonstrate that decreasing vimentin expression in the constitutively vimentin-expressing CL1 cells led to a significant decrease in their invasiveness but that forcing expression of vimentin in the LNCaP cells did not augment their invasiveness. These findings imply that vimentin expression contributes to the invasive phenotype but cannot confer it alone.

INTRODUCTION

With the advent of prostate-specific antigen testing for prostate cancer, this disease is increasingly being detected at a potentially curable stage. There remain, however, major challenges to the successful management of this, the most commonly diagnosed cancer in men in the Western world. Although the number of men diagnosed with extraprostatic disease is decreasing, a significant proportion of those treated with curative intent go on to develop advanced disease. For these patients, androgen ablation is the mainstay of treatment, and although the initial response rate approaches 80%, most patients demonstrate biochemical or clinical relapse within 18 months (1). Moreover, the heterogeneity of the disease, in which cancer cells within the same patient may have different histological features and metastatic potential, means that outcome after treatment is extremely unpredictable. At a fundamental biological level, very little is known about what makes the disease aggressive in some and relatively indolent in others. The purpose of this study was to elucidate factors making an androgen-independent prostate cancer cell line much more aggressive than the androgen-sensitive line from which it was derived.

The model used compared the androgen-sensitive LNCaP prostate cancer cell line and an aggressive, androgen-independent subline, CL1. The CL1 cell line was derived by continuous subculturing of LNCaP cells in media deprived of androgen by supplementation with charcoal-stripped serum (2). Although a number of molecular characteristics of the CL1 line have been documented, we sought to compare it with the parental line using a limited, human cancer-specific cDNA microarray. Among the differentially expressed genes between the two cell lines, one coding for an intermediate filament cytoskeletal protein, vimentin, was overexpressed 20-fold in the CL1 cells. Among actin filaments, microtubules, and intermediate filaments, the three major classes of cytoskeletal proteins found in eukaryotic cells, intermediate filaments are the most complex (3). There are around 50 types of intermediate filament proteins that are categorized into 5 subtypes, and vimentin belongs to type III.

Having established that vimentin expression was up-regulated in the transition of LNCaP to CL1 cells, our aim was to determine the importance of vimentin expression to the aggressive phenotype of prostate cancer cells. LNCaP cells were stably transfected with a vimentin sense cDNA construct, and CL1 cells were transfected with the corresponding antisense construct. An in vitro invasion assay using Matrigel reconstituted basement membrane was then used to test the resulting cell lines.

MATERIALS AND METHODS

Cell Culture. The androgen-responsive human prostate cancer LNCaP and nonresponsive DU145 and PC3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The MatLyLu anaplastic Dunning rat prostate cancer cell line was a generous gift of Dr. S. A. Rabbani (McGill University, Montreal, Canada). The cell lines were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 1% antibiotic-antimycotic solution (Life Technologies, Inc.), 10 mM HEPES, and 1.0 mM sodium pyruvate. The CL1 androgen-independent subline derived from the LNCaP line, a generous gift from Dr. A. S. Beldegrun (University of California Los Angeles Medical School, Los Angeles, CA), was maintained in RPMI 1640 supplemented with 10% charcoal-stripped FBS. Cells were kept at 37°C in a humidified atmosphere with 5% CO2.

DNA Microarray Analysis. Total RNA was extracted from LNCaP and CL1 cell lines using the Qiagen RNeasy Minikit. Radioactively labeled cDNA probes were created from total RNA and hybridized to Clontech human cancer cDNA nylon arrays containing 588 genes (Clontech, Palo Alto, CA) according to the manufacturer's instructions. After a series of washes, the hybridized membranes were exposed to a phosphorimager plate for 48 h and imaged using the Storm PhosphorImaging system (Molecular Dynamics Inc., Sunnyvale, CA). The signal intensity of each cDNA pair of spots was quantified using ImageQuant software (Amersham Biosciences) by subtracting the local regional background intensity from each spot. The two membranes were normalized to each other by using the signal of housekeeping genes provided.

Northern Blot Analysis. Total RNA was extracted separately for analysis by Northern blot. Ten μg of total RNA were separated on a RNA gel and transferred overnight to a nylon membrane (Boehringer Mannheim). A cDNA probe was created from a 1.1-kb vimentin clone obtained from the American Type Culture Collection (catalogue number 59160). The probe was labeled with [α-32P]dCTP (New England Nuclear, Boston, MA) using the RTS Radiolabeling system (Life Technologies, Inc.) and hybridized to the membrane. The membrane was washed after hybridization, exposed for 48 h to a phosphorimager plate, and imaged using the Storm PhosphorImaging system. The 18S and 28S ribosomal bands were used to assess equal loading of RNA.

Western Blot Analysis. Cells were treated with lysis buffer containing 15% NP40, 5 μM NaCl, 2 μM Tris base (pH 7.4), and 0.5 μM EDTA (pH 8.0). Protein concentrations were determined using the Bio-Rad protein assay. Equal amounts of protein denatured in SDS sample buffer [2% SDS, 62.5 mM Tris base (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue] were loaded onto 10% SDS-PAGE, and gels were transferred onto nitrocellulose membranes (Amersham Biosciences). Equal loading of protein...
was confirmed by staining the membrane with Ponceau S (Sigma). The membranes were blocked overnight in Tris-buffered saline containing 5% (w/v), skimmed milk powder and, after a series of washes, blots were stained with the recommended dilution of primary antibodies against vimentin (V9; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CK directed against CK7 and CK8 (Cam 5.2; Becton Dickinson), or β-actin (C2; Santa Cruz Biotechnology). After further washing, the blots were incubated with a 1:1000 dilution of goat antigoat secondary antibody (Horse-Rederabbit) and then washed in PBS. This was followed by a 1-h incubation with Alexa Fluor 488 goat antigoat secondary antibody (Molecular Probes, Eugene, OR). Nuclei were stained by a 10-min incubation with DAPI (Sigma).

Expression Vector Construction. A 1.8-kb vimentin cDNA fragment was excised from the pCMV-SPORT6 plasmid vector supplied by the American Type Culture Collection. This was cloned into the KpnI/NorI (sense orientation) and EcoRI/HindIII (antisense orientation) sites of pcDNA3.1 vector (InVitrogen, Carlsbad, CA).

DNA Transfection and Clone Selection. LNCaP cells were transfected with 3 μg of the pcDNA3 vector with the vimentin insert in the sense orientation, and CL1 cells were transfected with the vector containing vimentin in the antisense orientation, according to the protocol supplied with the LipofectAMINE transfection reagent (Life Technologies). Incubation with LipofectAMINE for 4 h, 2 × 10^5 cells were plated in 6-well dishes and incubated with the appropriate DNA and LipofectAMINE in serum-free media for 5 h, and then equal volumes of media containing 20% FBS were added. At 24 h, the media were replaced with media containing 1 mg/ml G418. Surviving colonies were selected after 2 weeks and then maintained in 350 μg/ml G418 (CL1 cells) or 185 μg/ml G418 (LNCaP cells). Changes in vimentin levels were assayed for by both Western blotting and immunofluorescence microscopy.

Motility and Invasion Assays. Cell motility was assessed using 24-well Biocoat Control Insert Chambers (Becton Dickinson) with polycarbonate filters containing 8-μm pores. Cells were plated at 1.0 × 10^5 cells/well (LNCaP) and 1.5 × 10^6 cells/well (CL1) in 0.5 ml of serum-free medium. The outer chambers were filled with 0.5 ml of media containing 10% FBS. After 48 h (LNCaP) or 24 h (CL1), cells migrating to the undersurface of the filters were counted. The top surface of the membrane was gently scrubbed with a cotton bud, and cells on the undersurface were fixed in methanol and stained with H&E before undergoing a series of washes. The same five microscopic fields were used to count the number of cells passing to the undersurface of each filter. For invasion assays, the control insert chambers were replaced with Biocoat Invasion Chambers (Becton Dickinson) containing a Matrigel reconstituted basement membrane layer.

RESULTS

Up-Regulation of Vimentin in CL1 Cells. The derivation of CL1 hormone-refractory prostate cancer cells from the parental hormone-sensitive LNCaP cell line has been described previously (2). The CL1 cells were shown to have an accelerated growth rate, resistance to radiation/cytotoxic anticancer drugs, and highly tumorigenic behavior even in castrated and female mice when compared with the parental cell line. In addition, up-regulation of particular molecules, including interleukin 6, fibroblast growth factor, and vascular endothelial growth factor, associated with aggressive cancer cell lines was sought and observed. Both LNCaP and CL1 cells expressed high levels of the epidermal growth factor receptor, although levels were higher in the CL1 cells. To establish whether other classes of molecules were involved in the acquisition of an aggressive, invasive phenotype, the cells were compared using the Clontech Atlas Human Cancer array. RNA was extracted from both cell lines, and radiolabeled cDNA probes were prepared for hybridization to the arrays. A 10-fold difference in expression was set as the minimum difference to be further investigated. The signal for one gene, vimentin, met this criterion, being overexpressed 20-fold in the CL1 cells with respect to the LNCaP cells. An image of a pair of hybridized cDNA membranes with the site of the cDNA spot for vimentin outlined on the membrane hybridized with CL1 RNA is shown in Fig. 1A.

The findings from the microarray were confirmed by Northern and Western analyses as shown in Fig. 1. B and C. Dual staining of LNCaP and CL1 cells with the vimentin V9 antibody and DAPI for nuclear staining is shown in Fig. 2. A and B. A network of vimentin intermediate filaments is clearly seen in the CL1 cells, whereas in the LNCaP cells, under the same conditions, only the nuclear staining is visible.

In addition, the expression of vimentin was sought in both DU145 and PC3 androgen-insensitive, invasive human prostate cancer cell lines as well as the MatLyLu rat prostate cancer cell line. High levels of vimentin in comparison with those seen in LNCaP cells are shown by Western analysis in Fig. 1C. Immunocytochemical staining of these cells (Fig. 2, G–I) demonstrates a dense network of vimentin filaments, similar to that seen in CL1 cells.

CK Distribution in LNCaP and CL1 Cells. Earlier reports of vimentin expression in invasive breast cancer cell lines have suggested that coexpression of vimentin and CK intermediate filaments is necessary to confer the invasive phenotype (3). Cells of epithelial origin would be expected to express CK intermediate filaments (3). A Western blot (Fig. 3) comparing amounts of CK7 and CK8 in the CL1 and LNCaP cells demonstrates higher levels in the LNCaP cells, although immunofluorescence microscopy (Fig. 2, C and D) shows that both cell lines do have a network of CK filaments.

Stable Transfection of Sense and Antisense Vimentin Constructs. Three clones of LNCaP cells transfected with the vimentin sense vector and four clones of CL1 cells transfected with the antisense vector were established by ring cloning. Protein levels of vimentin in the clones were assayed by Western blot as well as by immunofluorescence microscopy. The CL1 clone that demonstrated the lowest level of vimentin expression by Western blot

![Image](image-url)

Fig. 1. CL1 cells express higher levels of vimentin than the parental LNCaP cells. A, a pair of hybridized Atlas Human Cancer Array cDNA membranes. The site of the cDNA spot for vimentin is shown by an arrow on the membrane hybridized with RNA from CL1 cells. The signal intensity of the vimentin cDNA spot on the CL1 membrane was 20 times that seen on the LNCaP membrane. B, Northern blot comparing vimentin expression in CL1 and LNCaP cells. Equal loading of RNA was confirmed by staining of 28S and 18S ribosomal bands. C, Western blot comparing levels of vimentin in LNCaP cells with levels of vimentin in CL1, DU145, PC3, and MatLyLu cells. Equal loading of protein was confirmed by staining the blot with Ponceau S stain.

2307
Fig. 2. Immunocytochemical staining with vimentin V9 antibody. A, distribution of vimentin in CL1 cells. B, distribution of vimentin in LNCaP cells. Blue, nuclear staining with DAPI. A dense network of vimentin intermediate filaments is seen in the CL1 cells, whereas under the same conditions, only nuclear staining is seen in LNCaP cells. C and D, immunocytochemical staining of CL1 (C) and LNCaP (D) cells with Cam 5.2 anti-CK primary antibody. CK is seen in the CL1 cells, although a denser network of filaments is observed in LNCaP cells. E, decreased vimentin distribution and altered morphology in CL1-AS4 cells. F, vimentin network seen in LNCaP-S2 cells. DU145 (G), PC3 (H), and MatLyLu (I) cells demonstrating vimentin cytoskeleton.

(Fig. 4A) is designated CL1-AS4. These cells have a rounded morphology and loss of discrete vimentin filaments (Fig. 2E) when compared with the wild-type cells (Fig. 2A). Increased expression in the LNCaP clone with the highest levels of vimentin expression is shown by Western blot in Fig. 4B. This is demonstrated most graphically by immunofluorescence microscopy in Fig. 2F. Vimentin filaments that are not identifiable in the wild-type cells are clearly seen here.
Motility and Invasive Potential of Wild-type Cells Compared with Transfected Cells. The motility of cells was tested by counting the number of cells passing through an uncoated filter containing 8-μm pores in response to a serum gradient. To test invasive potential, a similar assay was performed with filters coated with reconstituted basement membrane (Matrigel). The motility and invasion assays were carried out on three filters for each cell line, and cells were counted from the same five fields of each filter. Growth curves comparing wild-type with transfected cells showed no difference in proliferation over the time course during which the assays took place (data not shown). Fig. 5A shows the motility of the wild-type and transfected cell lines represented as the mean number of cells counted on the undersurface of each filter per field counted. The motility of the transfected cells is not altered with respect to the wild-type cells under these conditions. The invasion assay data are presented in Fig. 5B. It is clear that in the CL1-AS4 cells, with decreased expression of vimentin, invasion through the Matrigel reconstituted basement membrane is effectively abolished ($P < 0.001$). However, when the wild-type LNCaP cells were compared with the transfected clone demonstrating the highest levels of vimentin (LNCaP-S2), no increase in invasive potential was seen. Both cell lines were unable to invade through the Matrigel membranes in appreciable numbers over the 48-h period in which the assay was carried out.

DISCUSSION

The aim of this study was to identify factors involved in the conversion of noninvasive, hormone-dependent prostate cancer to aggressive, hormone-independent cancer. The model used was an in vitro analogue of the situation seen during prostate cancer treatment, in which initially successful therapeutic hormone deprivation is followed by unresponsive, highly invasive disease. To identify differentially expressed genes, a cDNA hybridization analysis of 588 well-characterized genes covering a broad range of cellular functions was used. Other investigators have used microarrays bearing thousands of genes to compare prostate cancer cell lines and RNA from tissue samples to search for genetic differences (4–6). Our approach was directed at establishing differences emerging specific to the process of hormone deprivation and their effect on the invasive potential of prostate cancer cells. Given the heterogeneity of prostatic biopsy samples and the great differences between even cell lines established from the same organ, this targeted approach is valid. The gene demonstrating the greatest differential expression between the cell lines, vimentin, was selected for further study.

Early work suggested that the expression of intermediate filament proteins was tissue type specific, with normal and tumor tissue expressing a single class of intermediate filament (7). In this context, vimentin is considered to be the intermediate filament of mesenchymal tissue (8), and as such, it has been used as a tumor marker in the diagnosis of melanoma (9). Epithelial tissues and tumors have traditionally been characterized by their CK expression (10). However, coexpression of both vimentin and CK intermediate filaments has been shown in prostate cancer (11) and a variety of other tumor cell lines and tissues (12–15).

The coexpression of vimentin and CKs has been strongly associated with a more aggressive and metastatic phenotype in breast cancer. Data from breast cancer cell lines have demonstrated that ER-negative, aggressively behaving cell lines express vimentin, whereas ER-positive, noninvasive cell lines do not (13). In an immunohistochemical analysis of breast cancer specimens (16), vimentin immunopositivity was inversely related to keratin and ER expression but positively correlated to tumor progression. The distinction between these cell populations is further supported by clinical evidence. Breast cancer patients with tumors that do not express vimentin and are ER negative are less likely to experience local recurrence and distant metastases than those whose tumors express vimentin and are ER positive (17). The authors conclude that in breast cancer, vimentin and ER are coexpressed in tumors that are aggressive and resistant to hormone therapy, suggesting that vimentin and ER may serve as a biological marker to identify patients at greatest risk for unfavorable outcome. Therefore, the study of vimentin expression may provide useful information for selecting patients for early hormone therapy or for developing hormone therapy-resistant cell lines.
grade. Tumors in which vimentin and CKs were expressed in approximately similar amounts were those with the worst prognosis. Coexpression of keratins and vimentin is also associated with recurrent and metastatic disease in melanoma (15).

Our data demonstrate an increase in vimentin expression of the CL1 cells accompanied by a decrease in the levels of CKs. This type of change in the expression profile of intermediate filaments in epithelial cells is referred to as EMT. This transition of cell phenotype occurs physiologically during normal developmental processes that require cell migration and extracellular matrix invasion and during wound healing (17). EMT is now becoming well recognized as a hallmark of tumor progression, characterizing invasive and metastatic carcinomas (18). As well as a switch from CK to vimentin intermediate filament expression, EMT involves a reduction in expression of cell adhesion molecules, particularly E-cadherin. It has been proposed that EMT represents a permanent switch in certain tumors (19), and this was the finding in mammary epithelial cells that underwent EMT in response to transfection with the matrix metalloproteinase stromelysin 1 (20). This is consistent with the finding that the transition in phenotype undergone by CL1 cells is not reversible by replacing the charcoal-stripped serum in which they grow with untreated FBS (2).

Data to show that vimentin expression in prostate cancer is associated with a more aggressive phenotype have been contradictory. Our data confirm the findings of previous studies in which the phenotypes of prostate cancer cell lines have been examined and in which LNCaP cells show low levels of vimentin expression in contrast to the high expression seen in their more aggressive DU145 and PC3 counterparts (21, 22). In a differential hybridization analysis of rat prostate carcinoma sublines from the Dunning model, a protein with 96% homology to human vimentin was found to be highly expressed in all of the hormone-independent, anaplastic tumors. Its expression was very low in normal prostate and in the well- or moderately differentiated Dunning sublines. Our results using the anaplastic MatLyLu cells, the most aggressive of the Dunning sublines, corroborate these data. This finding was similar to that of the earlier study of breast cancer cell lines, in which hormone receptor status and invasiveness were considered with vimentin expression (13). A subsequent immunohistochemical study of 15 cases of tumor and 49 cases of nodular hyperplasia found vimentin expression in both tissues, but no correlation could be made between vimentin expression and tumor grade (23). This led to the conclusion that vimentin expression could not be used to help distinguish high-grade from low-grade tumors. However, a recent, more elaborate study concerned with factors associated with motility in prostate cancer cell lines and poorly differentiated metastatic prostate cancer did point to the importance of vimentin expression in prostate cancer (24).

Here it was reported that among a number of adhesion molecules, cytoskeletal elements, and prostate-specific markers, vimentin expression alone correlated with motility of prostate cancer cell lines. However, levels of vimentin expression did not necessarily correlate with invasiveness in all of the cell lines tested. Immunohistochemical studies showed that vimentin expression was found in poorly differentiated prostatic tumors and in prostate tissue from patients with metastatic disease identified by bone scan. In addition, bone metastases resulting from prostate cancer were analyzed, and the majority of these were shown to stain positively for vimentin.

Having established that vimentin expression was barely detectable in LNCaP cells and highly expressed in the faster-growing and more aggressive CL1 subline, we wanted to assess the contribution of vimentin to the invasive phenotype. We found that experimentally reducing the expression of vimentin in the CL1 cell line effectively abolished the invasive potential of CL1 cells in the Matrigel invasion assay. In experiments conducted with breast cancer cell lines (3), transient down-regulation of vimentin in MDA-MB-231 cells led to a decrease in their migratory ability that the authors considered to be indicative of decreased metastatic potential. The MDA-MB-231 cell line shares characteristics of the CL1 cell line in that both constitutively coexpress vimentin and CKs and are unresponsive to hormonal stimulation.

When vimentin expression was forced in LNCaP cells by stable transfection, we were not able to observe acquisition of an invasive phenotype using the Matrigel invasion assay. Similar experiments conducted in the MCF-7 breast cancer cell line have produced controversial results. This cell line, like the LNCaP cell line (25, 26), does not constitutively express vimentin, is hormone sensitive, and has a slow growth pattern in nude mice (27). Sommers et al. (28) were unable to increase the invasiveness of MCF-7 cells by stable transfection with vimentin, whereas Hendrix et al. (3) used a different expression vector and experimental conditions, were able to increase the invasive and tumorigenic potential in in vitro assays. However, artificial expression of vimentin in these cells was not sufficient to confer the metastatic phenotype of MDA-MB-231 cells when they were inoculated into severe combined immunodeficient (SCID) mice.

The immunohistochemical studies of vimentin in prostate cancer to date have been performed with a view to evaluating its expression as a marker for aggressive or metastatic disease. Although vimentin itself may not be the marker that investigators had hoped for, the experimental evidence surrounding vimentin and other intermediate filament proteins indicates that this is an important field in prostate cancer tumor biology and the development of metastases. The finding that vimentin expression is up-regulated in this model of androgen-deprived prostate cancer cells, in the hormone-refractory Dunning rat prostate cancer sublines (29), and in ER-negative breast cancer cell lines (15) raises the issue of its influence in the aggressiveness of hormone-independent disease. Recently, in a study considering the role of vimentin as a tool for cellular immortalization, 1757 bp of the human vimentin promoter were sequenced (30). Androgen response elements were not among the transcription factor binding sites identified, so it would seem that the effect of hormonal regulation on vimentin expression is an indirect one.

There is growing evidence that the extracellular matrix can regulate gene expression and that it does this via certain cell surface integrin receptors (31, 32). Importantly, intermediate filaments including vimentin seem to act as mechanical transducers between cell surface integrins and nuclei (33, 34). Furthermore, the finding that inappropriate expression of intermediate filament proteins may be involved in the conference of drug resistance (35) is of great relevance to the treatment of advanced, metastatic disease.

Our data, like those of others presented in different tumor types, suggest that constitutive coexpression of vimentin with CK in prostate cancer cell lines is associated with an invasive phenotype that can be reversed by reducing vimentin expression. However, it would seem that vimentin is likely to function with other proteins or is likely to act in one of the later stages in the invasion process, given the results seen in the LNCaP-S2 cells and previous data obtained from breast cancer cell lines (28). Additional studies to establish the function and interactions of vimentin in prostate cancer, particularly with regard to advanced, metastatic, and hormone-refractory disease, are warranted.

REFERENCES


Overexpression of Vimentin: Role in the Invasive Phenotype in an Androgen-independent Model of Prostate Cancer

Sadmeet Singh, Skanda Sadacharan, Scott Su, et al.


**Updated version**  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/63/9/2306](http://cancerres.aacrjournals.org/content/63/9/2306)

**Cited articles**  This article cites 34 articles, 11 of which you can access for free at: [http://cancerres.aacrjournals.org/content/63/9/2306.full#ref-list-1](http://cancerres.aacrjournals.org/content/63/9/2306.full#ref-list-1)

**Citing articles**  This article has been cited by 17 HighWire-hosted articles. Access the articles at: [http://cancerres.aacrjournals.org/content/63/9/2306.full#related-urls](http://cancerres.aacrjournals.org/content/63/9/2306.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.

**Permissions**  To request permission to re-use all or part of this article, use this link [http://cancerres.aacrjournals.org/content/63/9/2306](http://cancerres.aacrjournals.org/content/63/9/2306).

Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.