CLT1 Targets Bladder Cancer through Integrin α5β1 and CLIC3

Lynn M. Knowles1, James Zewe1, Gunjan Malik1, Anil V. Parwani2,3, Jeffrey R. Gingrich1,3, and Jan Pilch1,3

Abstract
High-grade non–muscle-invasive bladder cancer is commonly treated with Bacillus Calmette-Guérin, an immunotherapeutic that depends on fibronectin and tumor cell integrin α5β1 for internalization into bladder cancer cells. We previously showed that the anti-angiogenic peptide CLT1 forms cytotoxic complexes with fibronectin that are cooperatively internalized into proliferating endothelium through ligation of integrins and chloride intracellular channel 1. While CLT1 has no effect on mature, differentiated cells, we show here that CLT1 is highly cytotoxic for a panel of bladder tumor cell lines as well as a variety of cell lines derived from kidney, lung, breast, and prostate cancer. Paralleling our previous results, we found CLT1-induced tumor cell death to be increased in the presence of fibronectin, which mediated CLT1 internalization and subsequent autophagic cell death in a mechanism that depends on tumor cell integrin α5β1 and chloride intracellular channel 3 (CLIC3). This mechanistic link was further supported by our results showing upregulation of α5β1 and CLIC3 in CLT1-responsive tumor cell lines and colocalization with CLT1 in tumor tissues. Incubating tumor tissue from patients with bladder cancer with fluorescein-conjugated CLT1 resulted in a strong and specific fluorescence whereas normal bladder tissue remained negative. On the basis of its affinity for bladder tumor tissue and strong antitumor effects, we propose that CLT1 could be useful for targeting bladder cancer.

Introduction
Bladder cancer is the second most common and the third deadliest malignancy of the genitourinary tract, with a mortality of approximately 20% (1). The majority of patients (75%–85%) have non–muscle-invasive bladder cancer (NMIBC) at the time of diagnosis, which is typically treated with transurethral resection (TURBT; ref. 2). While TURBT is effective in patients with low-risk bladder cancer (i.e., small single tumors, noninvasive, and low-grade), the rate of recurrence and progression in patients with intermediate- or high-risk disease is considerable (3, 4). To reduce recurrence, TURBT is usually accompanied by an adjuvant intravesical treatment with chemother or immunotherapy (5, 6). However, despite these therapies, the rate of recurrence and progression is up to 70% and 30%, respectively (5). Other strategies to control bladder cancer include hyperthermia, photodynamic therapy, and diverse imaging strategies that are intended to improve tumor visualization during cystoscopy and TURBT (2, 7, 8). While some of these approaches (e.g., hyperthermia) have shown benefit in the short term, their efficacy of controlling bladder cancer over a long period of time is not clear (7). Together, these data underscore the continued need to develop efficient and well-tolerated adjuvant treatments for high-grade NMIBC.

The adjuvant treatment of choice for high-risk NMIBC is the intravesical application of live bacteria, Bacillus Calmatte-Guérin (BCG), which is superior in reducing the risk of recurrence compared with intravesical chemotherapy with mitomycin (9–11). BCG has also been shown to reduce the risk of progression, but it is not clear whether the treatment yields a significant survival advantage (12, 13). The 2 major drawbacks of BCG are nonresponse and a high rate of side effects (~90% of cases) ranging from irritative voiding, hematuria, and cystitis to BCG-induced sepsis (13, 14). While the majority of BCG-induced side effects are considered as nonsevere, it has been estimated that they cause nearly 30% of the patients to discontinue therapy (14). The mechanism of action for BCG is to induce an immune reaction that orchestrates the eradication of bladder tumor cells and treatment complications are a direct result of this response (15). As such, side effects correlate positively with the efficacy of BCG, whereas the lack of a sufficient immune response is predictive of treatment failure.

Using phage display, we previously identified a tumor-homing peptide, CLT1 that associates with clotted plasma in tumor interstitial spaces (16). More recently, we found that CLT1 has strong anti-angiogenic activity in vivo and
attributed this function to the capacity of CLT1 to induce an unfolded protein response and autophagic cell death in proliferating endothelial cells (17). Cytotoxicity toward endothelial cells was supported by fibronectin, which forms co-aggregates with CLT1 and, as such, mediates binding to endothelial integrins and subsequent internalization through a novel CLT1 receptor, chloride intracellular channel 1 (CLC1; ref. 17). This mechanism is reminiscent of the role of fibronectin for BCG opsonization, which contributes to the integrin-mediated internalization of fibronectin-bound BCG into bladder tumor cells as a prerequisite of BCG antitumor activity (18, 19). On the basis of the role of fibronectin for BCG antitumor efficacy, we sought to determine whether CLT1 has antitumor properties and if this function is pronounced in bladder cancer.

Materials and Methods

Peptides

All peptides were purchased from Primm Biotech. A lysine/alanine scan of CLT1 yielded CLT1 variant peptides GA (CGLIQQKNEC), LK (CGAIQQKNEC), IK1 (CGLAIQQKNEC), IK2 (CGLIQQKNEC), QA (CGLIQQKNEC), KA (CGLIQQKNEC), NA (CGLIQQKNEC), and EA (CGLIQQKNEC). CLT1 peptides were cyclized by exposure to air, which leads to the formation of intramolecular disulfide bonds between C- and N-terminal cysteines. Linear CLT1 (LCLT1, 4GGLIQQKNEC) was generated by replacing the cysteines with alanine. Carboxy-fluorescin was conjugated to the peptides via a 2-aminoethyl-2-ethoxycarbonyl (AEEA) linker for fluorescent studies.

Cell lines and treatments

J82, T24, UMUC3, TCCSUP, 5637 (bladder), DU145, PC-3 (prostate), RCC4, 786-O (renal), MDA-MB-231, MCF-7 (breast), A549 (lung), PANCl (pancreas), and RD (muscle) tumor cell lines were purchased from American Type Culture Collection and cultured per manufacturer’s specifications. Human bladder epithelial cells were from Lifeline Cell Technology and cultured according to manufacturer recommendations in serum-free Prostalife Basal Medium supplemented with 1-glutamine, extract P, epinephrine, rh TGF-α, hydrocortisone hemisuccinate, rh insulin, apo-transferrin, and calcium chloride. Cells were cultured at 37°C under a humidified 5% CO2 atmosphere. All cells were treated at a plating density of about 50% to ensure a linear growth rate. Peptides were diluted in H2O to 2 mg/mL and added to cells at concentrations ranging from 7.5 to 150 μg/mL in the presence of 2% FBS. Human plasma fibronectin (Sigma; 3–100 μg/mL), fibrinogen (Enzyme Research Laboratories; 30 μg/mL), nocardazole (Sigma; 10 μmol/L), GRGDSP (RGD) or GRADSP (RAD) peptides (each 300 μmol/L; EMD Chemicals) were added at the time of peptide treatment. Bafilomycin A1 (EMD Chemicals; 40–400 nmol/L) was added 1 hour before CLT1 addition. Where indicated, FBS was depleted of fibronectin by passing the serum through a gelatin agarose column. For experiments using serum-free media, cells were serum starved for 8 hours before study onset.

Cytotoxicity assay

Cell death was assessed after 24 hours by measuring lactate dehydrogenase (LDH) release using the Cytotoxicity Detection Kit (Roche Applied Science). Results are normalized for background cell death observed in control cells treated in absence of CLT1 to the specified medium conditions. Maximal cell death was achieved by simultaneous treatment with camptothecin (1.4 μmol/L; Sigma-Aldrich) and staurosporine (100 nmol/L; Alexis Biochemicals).

Microscopy

To analyze internalization of CLT1 in tumor cell lines, cultured cells grown on coverslips coated with 10 μg/mL vitronectin (BD Biosciences) overnight at 4°C were incubated with 25 μg/mL fluorescein-conjugated peptides for 24 hours in the presence of 30 μg/mL fibronectin or fibrinogen where indicated, fixed in 4% paraformaldehyde (PFA) and stained with 4’,6-diamidino-2-phenylindole (DAPI)-containing mounting media ( Vectashield; Vector Laboratories). Cellular localization was analyzed at ×40 magnification using a fluorescence microscope (Zeiss Axioplan 2) with image processing unit. Lysosomes were labeled with 100 nmol/L Lysotracker Red DND-99 (Invitrogen) for 1 hour before fixation. For fibronectin and CLIC3 staining, PAA-fixed cells were permeabilized with 0.5% Triton-X100 and incubated with anti-fibronectin (Millipore), anti-CLIC3 (Abcam), or isotype control, followed by incubation with Alexa Fluor 546–conjugated secondary antibody (Invitrogen) and analyzed using a confocal microscope (Leica TCSSSL). Cell morphology was imaged by reflection confocal microscopy. To visualize nuclei, cells were stained with Draq5 (eBioscience).

De-identified tissues from clinical bladder cancer as well as normal adjacent bladder tissue were inspected by an anatomic pathologist following resection and then immediately distributed through the tissue bank of the University of Pittsburgh Cancer Institute (Pittsburgh, PA). Upon receipt in our laboratory, tissue was placed in keratinocyte growth media containing bovine pituitary extract, hEGF, insulin, hydrocortisone, and antibiotics with or without 25 μg/mL fluorescein-conjugated CLT1 or control peptides (IK1, LCLT1). Fibrinogen and fibrinogen were added where indicated. After overnight incubation at 37°C and 5% CO2, tissue samples were washed in PBS and either placed on a microscopy slide for en-face confocal microscopy or frozen in optimum cutting temperature (OCT). Frozen tissues were sectioned, fixed in acetone, and analyzed for peptide uptake by fluorescence microscopy. For immuno-histochemistry, acetone-fixed sections were incubated with anti-CLIC3 or anti-α5 integrin (BD Biosciences) and Alexa Fluor 546–conjugated secondary antibody before confocal microscopy. Digitized images were processed with Adobe Photoshop.
Western blot analysis

Cells were lysed using the Subcellular Protein Fractionation Kit (Thermo Fisher Scientific) or by the addition of ×2 SDS sample buffer. Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF), and stained with 0.05% Ponceau S (Sigma) to ensure equivalent protein loading. Immunoblots were blocked with 5% bovine serum albumin and probed overnight at 4°C with anti-Cathepsin D, anti-LC3B (Cell Signaling Technology) or anti-CLIC3. Immunoreactivity was detected using peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody and visualized by enhanced chemiluminescence.

siRNA-mediated gene silencing

Integrin α5 (L-008003-00), integrin β3 (L-004124-00), CLIC1 (L-009530-00), CLIC3 (L-011805-00), CLIC4 (L-013553-00), CLIC5 (L-020570-00), and nontargeting control (D-001810-10) On-TARGETplus SMARTpool siRNAs were purchased from Dharmacon. Cells were transfected with 10 nmol/L siRNA in Opti-MEM medium and incubated for an additional 43 hours before treatment with CLT1. Target knockdown was confirmed by reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis (Supplementary Fig. S1).

Flow cytometry assay

Cells were suspended in media supplemented with 2% FBS and incubated with integrin α5 or isotype control antibody (BD Biosciences) for 30 minutes at 4°C, washed with ice-cold media, and incubated for 30 minutes on ice with Alexa Fluor 488 anti-mouse F(ab’)2 (Invitrogen). To measure CLIC3 expression, suspended cells were incubated on a rotor for 45 minutes at room temperature before incubation with CLIC3 antibody for 30 minutes at 4°C. Cell viability was monitored by staining cells with 5 μg/mL propidium iodide (PI; Roche Applied Science). Fluorescence was examined on 10,000 viable cells per sample using a tabletop cytometer (Accuri C6).

Statistical analysis

Data were analyzed using unpaired 2-tailed Student t test or one-way ANOVA followed by the post hoc Tukey multiple comparisons test (GraphPad Prism 5). Treatment differences with a 2-sided P < 0.05 were considered significantly different. Error bars show mean ± SEM.

Results

CLT1 induces tumor cell death in cooperation with fibronectin

We previously showed that CLT1 has significant anti-angiogenic activity, which correlates with the ability of CLT1 to generate cytotoxic complexes with the adhesion protein fibronectin (17). Here, we show that CLT1 is cytotoxic for a diverse panel of human tumor cell lines derived from the bladder, prostate, kidney, breast, and lung (Fig. 1A). The cytotoxic activity of CLT1 was strongly augmented in the presence of fibronectin, which alone had no effect on the tumor cell fate (Fig. 1A and B; Supplementary Fig. S2A). Cytotoxicity in proliferating tumor cells occurred within 24 hours of treatment at a fibronectin concentration of 10 μg/mL and reached a plateau at a CLT1 concentration of 75 μg/mL (Fig. 1C and D). A second plasma adhesion protein, fibrinogen, had the opposite effect and effectively prevented CLT1-induced cytotoxicity (Fig. 1A). Among the different tumor types tested, CLT1 activity was pronounced in bladder cancer cells, which responded consistently with extensive cell death even in the presence of urine (Fig. 1A; Supplementary Fig. S2B). CLT1 was significantly more effective in proliferating than in serum-starved bladder tumor cells and largely inactive in primary bladder epithelial cells (Fig. 1A and B). To further dissect the role of fibronectin for bladder tumor cell death, we treated TCCSUP, J82, and UMUC3 cells with fibronectin-depleted serum, which in comparison to plasma fibronectin–supplemented serum was significantly less effective in mediating CLT1-induced cell death (Fig. 1E and F). Tumor cell death in response to CLT1 was also reduced when we transfected bladder tumor cells with siRNA against fibronectin; however, this was only the case when tumor cells were treated in absence of exogenous fibronectin, suggesting that CLT1 interacts with cellular fibronectin as well as plasma fibronectin (Fig. 1F). Disabling structural features of CLT1 that are relevant for fibronectin complex formation, such as the LIIQK motif or the C- and N-terminal cysteines that mediate cyclization (17), rendered CLT1 inactive (Fig. 1G). Together, our results show that CLT1 has substantial antitumor activity, which is pronounced in bladder tumor cell lines, and that this activity depends on an intact LIIQK motif as well as the presence of fibronectin.

Tumor cells undergo autophagic cell death in response to CLT1

To further delineate the mechanism of CLT1 cytotoxicity, we incubated tumor cells with fluorescein-conjugated CLT1 and analyzed its subcellular localization using fluorescence and confocal microscopy. These experiments showed that CLT1 uptake was significantly higher in J82 and UMUC3 bladder cancer cells that are sensitive for CLT1-induced cell death than in 786-0 kidney cancer cells that are largely resistant toward CLT1 (Fig. 2A; Supplementary Fig. S3). Conversely, CLT1 cytotoxicity was significantly reduced when we inhibited CLT1 uptake with nocodazole (Fig. 2B and C). Cos staining with lysotracker revealed that CLT1 was transported into lysosomes, which appeared enlarged and dysformed after treatment with CLT1 compared with untreated cells (Fig. 2D and E). To determine whether CLT1 induces lysosome dysfunction, we analyzed the cytoplasmic fraction of CLT1-treated tumor cells and found increased concentrations of the lysosomal protease cathepsin D as an indicator for lysosome leakage (Fig. 3A). In addition, we found that CLT1 causes LC3 conversion, indicating that sorting of CLT1 into lysosomes is mediated in the context of autophagy (Fig. 3B). Moreover, inhibiting fusion of autophagosomes and lysosomes with the autophagy inhibitor bafilomycin A1 significantly reduced CLT1 cytotoxicity in a panel of bladder and
prostate tumor cells (Fig. 3C). Together, our results show that tumor cells internalize CLT1 and that this process leads to lysosome dysfunction and autophagic cell death.

CLT1-induced cell death correlates with fibronectin-mediated internalization of CLT1

An important function of fibronectin is to form complexes with CLT1 that promote CLT1 uptake into the angiogenic endothelium (17). Paralleling these results, we show that fibronectin, which colocalized with CLT1 inside the tumor cells, supports CLT1 uptake into bladder and prostate tumor cells whereas fibrinogen largely prevents CLT1 internalization (Fig. 4A and B). Moreover, CLT1 internalization was significantly reduced in the presence of a peptide GRGDSP that blocks the RGD recognition motif of fibronectin-binding integrins but not in the presence of an inactive
control integrin with the RGD peptide or knocking down integrin α5, which binds to fibronectin in an RGD-dependent manner, also inhibited the cytotoxic function of CLT1, whereas knockdown of another fibronectin-binding integrin, αvβ3, had no effect on CLT1-mediated tumor cell death (Fig. 4D and E). Flow cytometry showed that CLT1-sensitive tumor cell lines generally expressed higher levels of integrin α5β1 on their cell surface than tumor cell lines that were resistant toward CLT1 treatment (Fig. 4F, Supplementary Fig. S3A). Together, these results indicate that CLT1 internalization and cytotoxicity is largely determined by interactions of CLT1–fibronectin complexes with tumor cell integrin α5β1.

CLT1 cytotoxicity is mediated by CLIC3

We previously showed that CLT1–fibronectin complexes are taken up into angiogenic endothelium through interaction with integrin αvβ3 and CLIC1 and that this interaction induces autophagic cell death (17). To determine whether CLIC proteins cooperate with fibronectin-binding integrins in mediating CLT1 cytotoxicity, we transfected tumor cells with siRNA against various CLIC family members before treatment with CLT1. While knocking down CLIC1, 4, and 5 had no effect, we found that siRNA against CLIC3 significantly reduced CLT1-mediated cell death in a panel of bladder cancer cell lines as well as DU145 cells (Fig. 5A and B). Subcellular fractionation and flow cytometry revealed that CLIC3 is expressed in the tumor cell membrane and extends from there to the cell surface (Fig. 5C and D). Importantly, CLIC3 colocalized with CLT1 inside tumor cells, suggesting a mechanistic link between CLIC3 and CLT1 internalization (Fig. 5E). A connection between CLIC3 and CLT1 became further apparent by analyzing total CLIC3 protein expression levels, which were overall increased in CLT1-responsive when compared with nonresponsive cell lines (Fig. 5F). Together, these results suggest that CLIC3 plays an important role for mediating the cytotoxic activity of CLT1.

CLT1 binds to tumor tissue from bladder cancer patients

To determine whether CLT1 interacts with clinical bladder cancer, we incubated tumor tissue from 7 patients with bladder cancer with fluorescein-conjugated CLT1 overnight and subsequently analyzed the samples by fluorescence and confocal microscopy. Corresponding hematoxylin and eosin (H&E)-stained sections were analyzed by a clinical pathologist for morphologic confirmation of tumor (Supplementary Fig. S4). A summary of patient characteristics is
provided in Supplementary Table S1. Fluorescence microscopy revealed that CLT1 was strongly taken up in freshly resected, unfixed tumor tissues from both non-muscle- and muscle-invasive bladder cancer (Fig. 6A and B). Notably, there was no detectable fluorescence in normal bladder tissue treated with CLT1 or in tumor tissues treated with an inactive CLT1 variant as control peptide indicating that the interaction of CLT1 with bladder cancer is highly specific. Additional analysis with confocal microscopy showed staining of single cells, indicating that CLT1 is internalized in a manner similar to what we observed in cultured tumor cells (Fig. 6C). CLT1 binding was more efficient when tumor tissues were cultured in the presence of plasma fibrinogen than in fibrinogen (Fig. 7A). The role of fibronectin for CLT1 binding was further supported by our finding that CLT1 colocalized with integrin α5 and CLIC3 in bladder tumor tissue sections (Fig. 7B). Together, our results show that CLT1 binds to clinical bladder tumor tissue and that CLT1 binding is pronounced in areas that are rich in integrin α5β1 and CLIC3.

Discussion

We previously showed that CLT1 forms aggregates with fibronectin that are cytotoxic for angiogenic endothelium (17). Here, we show that CLT1 is strongly cytotoxic for tumor cells and attribute this activity to interactions between CLT1–fibronectin complexes and the fibronectin receptor.
integrin α5β1, which mediates CLT1 internalization and autophagic cell death in cooperation with CLIC3. Interestingly, CLT1 cytotoxicity is particularly pronounced in bladder tumor cell lines that express large amounts of integrin α5β1 and CLIC3. Paralleling this result, we show that CLT1 binds strongly and specifically to clinical bladder cancer tissues that express integrin α5β1 and CLIC3.

CLT1 (CGLIIQKNEC) contains a unique hydrophobic peptide sequence, LIIQK, that is critical for its antitumor activity.
in return lead to the generation of a plethora of factors that promote tumor cell proliferation, survival, and invasion (26). Moreover, pro-invasive and anti-apoptotic factors alter the interaction of integrins with the extracellular matrix by increasing turnover and recycling of activated integrins that are subsequently available for binding and internalization of CLT1–fibronectin complexes (27, 28).

The interaction between integrin α5β1 and fibronectin appears to be a major determinant of CLT1 cytotoxicity because eliminating exogenous fibronectin in prostate tumor cells or replacing fibronectin with fibrinogen effectively antagonized CLT1 internalization and subsequent cell death. While there was minimal cell death in prostate tumor cells in the absence of plasma fibronectin, we achieved only partial inhibition of cytotoxicity in bladder tumor cells treated with CLT1 in plasma fibronectin-depleted media. Moreover, transfection with fibronectin siRNA caused additional reduction of CLT1-induced cell death, suggesting that bladder tumor cells generate endogenous fibronectin to support CLT1 internalization. This is in line with previous results showing that fibronectin is a significant component of urothelial tumor stroma (29, 30). Notably, for the purpose of CLT1 internalization, the origin of fibronectin appears to be irrelevant, as both plasma fibronectin and cellular fibronectin are able to interact with integrin α5β1 through their RGD-containing integrin recognition motif located within the fibronectin repeat III10 (31). More importantly, the interaction of CLT1–fibronectin complexes with α5β1 is preserved in the presence of urine, which is an important consideration for the intravesical treatment of bladder cancer in vivo.

In addition to fibronectin, we identified CLIC3 as an important factor for CLT1 cytotoxicity. However, while involved in CLT1 internalization and cytotoxicity, CLIC3 was unable to compensate for the loss of fibronectin, indicating that CLIC3 function depends on RGD-dependent ligation of α5β1 with the fibronectin component of the CLT1–fibronectin complexes. This is in agreement with a recent report showing that CLIC3 mediates internalization and recycling of integrin α5β1 from fibronectin (21). Endocytosis of fibronectin is important for the turnover of focal contacts and recycling of unligated integrin α5β1 that mediates adhesive interactions with fibronectin during cell migration and invasion (21, 32). As a consequence, factors that promote recycling of activated α5β1 such as CLIC3 are upregulated in invasive tumor cell phenotypes (21). On the basis of this, we propose a model where amyloidogenic CLT1–fibronectin co-aggregates co-opt a CLIC3-dependent recycling mechanism for integrin α5β1 in invasive tumor cells, thereby inducing lysosome dysfunction and autophagic cell death. Considering the high degree of homology between CLIC3 and the CLT1 receptor CLIC1, this mechanism could also include direct interactions of CLT1 with cell surface CLIC3 (33).

The mechanism of CLT1 uptake shares similarities with the integrin α5–dependent internalization of BCG–fibronectin complexes into bladder tumor cells, suggesting that CLT1 could be useful for detection and treatment of clinical
bladder cancer (18). This concept is further supported by our results showing that CLT1 is particularly effective in inducing cell death in bladder tumor cell lines that exhibit high levels of integrin α5β1. In addition, we show that CLT1 binds to human bladder tumor tissue ex vivo in the presence of integrin α5β1, which has been shown to be upregulated in high-grade bladder cancer (23). On the basis of these data, we conclude that CLT1 function depends on receptors that are commonly expressed in bladder cancer. Both BCG and CLT1 induce autophagy (17, 34); however, while internalization of BCG has no direct effects on the tumor cell fate (35), we show here that CLT1 causes autophagic cell death in bladder cancer cells. The antitumor mechanism of CLT1 is also different from apoptosis-inducing chemotherapeutics such as mitomycin C, suggesting that CLT1 could be effective as a second line treatment or in combination with BCG, which has been shown to render mitomycin C inactive (35). Overall, considering the robust antitumor activity of CLT1 in combination with its specific labeling of bladder tumor tissue, it will be interesting to determine whether CLT1 can make a significant contribution to the diagnosis and treatment of bladder cancer in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Authors’ Contributions
Conception and design: L. Knowles, J. Pilch
Development of methodology: L. Knowles, J. Pilch, A.V. Parwani, J.R. Gingrich
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Knowles, G. Malik, A.V. Parwani, J.R. Gingrich, J. Pilch
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): L. Knowles, J. Zewe, G. Malik, A.V. Parwani, J.R. Gingrich, J. Pilch
Writing, review, and/or revision of the manuscript: L. Knowles, J. Zewe, G. Malik, A.V. Parwani, J.R. Gingrich, J. Pilch
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Knowles, J. Zewe
Study supervision: J. Pilch

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