Changes in Tumor Growth and Metastatic Capacities of J82 Human Bladder Cancer Cells Suppressed by Down-Regulation of Calreticulin Expression

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**In the United States, bladder cancer was among the top 10 most frequently diagnosed cancers in 2009.† The most common type of bladder cancer is transitional cell carcinoma, a malignant tumor that grows from the epithelium of the bladder. Approximately 30% of patients present with a muscle-invasive metastasis at the time of diagnosis, and 85% of these patients will die of the disease within 2 years if left untreated.‡ This means that metastasis is one of the leading causes of cancer-related death for patients with bladder cancer. Tumor cells detach, invade, and disseminate to other sites and form secondary tumors during metastasis. Each step involves many molecules, including cell-adhesion molecules, matrix degradation enzymes, and various growth factors.¶ However, the gene that regulates metastasis in bladder cancer is still unclear.

Calreticulin (CRT) is a 46-kDa multifunctional molecular chaperone protein in the endoplasmic reticulum (ER) that is conserved in various species.¶ CRT functions have been implicated in Ca2⁺ homeostasis, signaling transduction, gene expression, and glycoprotein folding.¶ Supported by the Frontier and Innovative Research Division of National Taiwan University (98R0318) and by grants (NSC97-2311-B-002-002-MY3 and NSC99-2120-M-002-004) from the National Science Council of the ROC (H.L.).

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Many studies elucidated that the alteration of CRT levels affects cell adhesion ability.8–10 Cells with overexpressed CRT have increased adhesiveness; in contrast, knockdown of CRT suppresses adhesion.11–13 Moreover, it was also reported that activation of matrix metalloproteinase 2, which plays an important role in cancer invasion and metastasis, increases as a result of CRT overexpression in rhabdomyosarcoma cells.14

In some cancers, tumor tissues express higher levels of CRT compared with normal tissues. CRT expression is significantly up-regulated in hepatoma, prostate, colon, and vaginal cancers.15–18 Furthermore, not only do CRT levels increase in urinary cancer tissues, but they also act as a biomarker for bladder cancer.19,20 In addition to being overexpressed in tumor tissues, CRT also regulates cancer cell behavior. Chen et al21 showed that the overexpression of CRT overexpressed in tumor tissues, CRT also regulates cancer progression of bladder cancer.22 In this study, we established CRT overexpressed and knockdown stable cell lines in J82 cells to clarify the role of CRT on progression of bladder cancer. Two months later, antibiotic-resistant single clones were selected and amplified to test CRT mRNA and protein expressions. The inducible YFP, ss-YFP-CRT cell lines (%5y and %5yCRT) were pretreated with 1 μg/mL tetracycline for 48 hours to perform the experiments.

**Transfection and Selection of Stable Cell Lines**

For transfection experiments, J82 cells were plated 2 × 10^6 cells/well in 6-well plates, and 2 μg of plasmid DNA for CRT overexpression (%5y and %5yCRT) or knockdown (pCR3.1 and CRT-RNAi) was transfected into cells with the use of 10 μL of Lipofectamine TM2000, (Invitrogen). For single-cell selection, the transfected J82 cells were passed at a 1:10 dilution in 10-cm culture plate at 2 days after transfection, and the stable cells were picked from a single colony with the use of cloning rings. Medium with antibiotics (200 μg/mL Hygromycin for %5y and %5yCRT inducible clones and 700 μg/mL G418 for pCR3.1 and CRT-RNAi stable clones) was replaced every 2 or 3 days. Two months later, antibiotic-resistant single clones were selected and amplified to test CRT mRNA and protein expressions. The inducible YFP, ss-YFP-CRT cell lines (%5y and %5yCRT) were pretreated with 1 μg/mL tetracycline for 48 hours to perform the experiments.

**Materials and Methods**

**Cell Culture**

The J82 human bladder cancer cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum under a humidified atmosphere of 95% air and 5% CO_2_ at 37°C. For the subcultures, cells were trypsinized with 0.05% EDTA-trypsin.

**Construction of Inducible Cell Lines**

To set up a tetracycline-inducible CRT system, the pEYFP-N1 was subcloned into pcDNA5/TO (Invitrogen, Camarillo, CA) by KpnI and NotI to generate pcDNA5/TO-YFP (%5y) used as control. CRT was amplified from HEK293 cDNA and subcloned into pcDNA5/TO-YFP to generate pcDNA5/TO-ss-YFP-CRT (%5yCRT) by the following primers: ss-forward-Nhel, 5′-CCGCTAGCTTGATGCTGATCCGT-3′, ss-reverse-HindIII, 5′-CCAGGCTTGGCTGACGGGAGCCAGGA-3′; YFP-forward-HindIII, 5′-CCAAGCTTGGCTGACGGGAGCCAGGA-3′, YFP-reverse-KpnI, 5′-AAGGTACCTTGTGACAGACGTGCTCAGTCACGCTCCG-3′; CRT-forward-KpnI, 5′-AAGGTACCGAGCCGCGCTCTACTTCAA-3′, and CRT-reverse-Xbal, 5′-AATCTAGACTACGCTGTCCTTGGCCCTGG-3′.

**Image Analysis**

For analysis of cell shape, morphologic images of J82 cells were thresholded and outlined with the use of MetaMorph software (Sunnyvale, CA). Changes in cell shape were assessed by a shape factor (sf = 4A/p^2) calculated from the area and the perimeter of the delineated object. Value close to “1” represents a flat object, whereas a value close to “0” represents a circular object.

**Immunoblotting**

Cells were plated at 2 × 10^5 cells/well in six-well plates overnight. For harvesting cell lysates, cells were washed with cold PBS and lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.0), 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS (pH 7.4)] containing protease inhibitors. Equal amounts of each sample (30 μg) were boiled with 6× sample buffer for 5 minutes and resolved by 10% SDS-PAGE. After electrophoresis, the gel was transferred to a polyvinylidene difluoride membrane. Membranes were blocked by 5% bovine serum albumin in 10 mmol/L Tris, 150 mmol/L NaCl, and 0.1% Tween-20 (pH 7.4) at room temperature for 2 hours. Blocked membranes were incubated with the following primary antibodies: CRT, Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA); paxillin (pY118), Cell Signaling (Danvers, MA); focal adhesion kinase (FAK; pY397), Invitrogen; FAK, paxillin, c-Src, and actin, Santa Cruz Biotechnology; and Src (pY418), Biosource, for 2 hours or 4°C overnight. Membranes were washed and then incubated with horseradish peroxidase–conjugated second-
ary antibodies (1: 5000) for 1 hour. Immunoreactive bands were quantified with TotalLab software version 2.01 (Durham, NC).

**RNA Isolation and Real-Time PCR**

Total RNA were isolate by the TRizol reagent, following the manufacturer’s instructions. RT-PCR was performed with ReverTra Ace reverse transcriptase. Real-time PCR was performed with the iCycle IQ real-time detection system (Bio-Rad, Hercules, CA) with the DNA double-strand specific SYBR Green I dye for detection. Cycling condition was 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. For quantification, the target gene was normalized to gliceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal control gene. Primer sequences were forward 5'-GTTGTCTCTGACTCAAC-3' and reverse 5'-TCTCTTCCCTTTGCTTGCTG-3' for GAPDH and forward 5'-AAATCTACGGTGACGGAGG-3' and reverse 5'-GTCGATTTCTGCTACGTTC-3' for CRT.

**Cell Cycle Analysis and Proliferation Assay**

Cells were starved overnight and then treated with cultured medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum) for 10 hours. Cells were trypsinized and resuspended in 1 mL of PBS. Propidium iodide was added to the suspensions and then incubated in a 37°C water bath for 20 minutes in the dark. Propidium iodide–stained cells were analyzed by flow cytometry, and the percentages of different phases were automatically analyzed by Partec FloMax software (Münster, Germany). To estimate cell proliferation rate, cells were plated in triplicates at 2500 cells/well in 96-well plates. Ten microliters of 5 mg/mL MTT was added to each well and incubated at 37°C for 20 minutes. After incubation for 4 hours at 37°C, MTT-containing medium was removed. MTT metabolic products were subsequently dissolved in 100 µL of dimethyl sulfoxide for 20 minutes at 37°C, followed by measurement of absorbance at 550 nm.

**Cell Migration Assay**

Migration rates of different cell lines were assayed in a modified Boyden’s chamber (Neuro Probe, Gaithersburg, MD). Cells suspended in 50 µL (1.5 × 10⁴ cells/well) were loaded in the upper chamber. Dulbecco’s modified Eagle’s medium and fetal bovine serum were loaded in the lower chamber as the negative control and chemoattractant, respectively. After 4 hours, migrating cells were fixed and quantified by a colorimetric measurement with the use of crystal violet staining with TotalLab v2.01 software.

**Cell Attachment Assay**

The 96-well culture plates were coated with 10 µg/mL collagen I and incubated at 37°C for 30 minutes followed by a PBS wash. Cells (5 × 10⁴ cells/100 µL) were seeded in the well and incubated at 37°C for 20 minutes. After removing the medium and non-attached cells, wells were washed with PBS, and 0.1% crystal violet was added for 10 minutes. The plate was gently washed with PBS three times. Then 10% acetic acid was added for 20 minutes, and the plate was read at 550 nm.

**Apoptosis Detection Assay**

Cells were starved overnight and treated with 5 µg/mL actinomycin D (Sigma, St. Louis, MO) as positive control for 24 hours. Cell apoptosis rate was detected with the use of fluorescein isothiocyanate Annexin V apoptosis detection kit I (BD Pharmingen, San Diego, CA). Cells were trypsinized and resuspended in 100 µL of 1× binding buffer. Fluorescein isothiocyanate, Annexin V, and propidium iodide (5 µL) were added to the suspensions and then incubated for 20 minutes at room temperature in the dark. Annexin V–stained cells were analyzed by flow cytometry. To measure end-stage apoptosis, TUNEL labeling was performed in tumor sections of the two groups with the use of a DNA fragmentation detection kit (Calbiochem, Gibbstown, NJ), following manufacturer’s instructions. Each section had been chosen for five images, and TUNEL-positive cells were quantified under microscope by high-power field (magnification, ×200). For a positive control, sections were incubated in DNase I before the addition of equilibration buffer, whereas double distilled water was used instead of terminal deoxynucleotide transferase reaction mix in the negative control.

**Subcutaneous Injection Model**

For models of bladder cancer to measure tumorigenicity, 8-week-old male BALB/c nude mice (n = 10; National Defense Medical Center, Taipei, Taiwan) were housed in pathogen-free conditions and acclimatized for 1 to ~2 weeks. Mice were randomized to one of two groups and were subcutaneously injected with vector control (n = 5) and CRT-RNAi (n = 5) cells in the inguinal region. Cells (1 × 10⁷) were suspended in PBS and Matrigel in a 1:1 (v/v) ratio. Tumors were measured every 3 days (width² × length × 0.5). Mice were sacrificed after 45 days, and subcutaneous tumors were surgically excised.

**Tail Vein Injection Model**

For the metastasis model, 8-week-old male severe combined immunodeficient mice (n = 12; BioLASCO, Taipei, Taiwan) were used for tail vein injection. Cells (10⁵) suspended in PBS were injected through the tail vein. After 40 days, the lungs and liver were resected and fixed in 3.7% formalin for paraffin embedding in preparation for immunohistochemical analysis. The percentage of the lung metastasis area was calculated as the metastatic lung area/total lung area.

**Statistical Analysis**

Data were statistically analyzed with one-way analysis of variance, followed by Fisher’s protected least-significant difference test (StatView; Abacus Concept, Berkeley,
CA). Each result was obtained from at least three independent experiments, and a value of $P < 0.05$ was considered statistically significant.

Results

Generation of Stable Cell Lines

To clarify the effects of CRT in bladder cancer cells, we attempted to select CRT overexpression and knockdown stable cell lines. J82 bladder cancer cells were transfected with a CRT overexpressed plasmid and a CRT-RNAi plasmid and then selected by respective antibiotics. After selection by G418 over 2 months, CRT-RNAi cells were generated, and CRT knockdown efficiency was confirmed with a real-time PCR and Western blotting to detect mRNA (Figure 1B) and protein (Figure 1C) expression levels compared with the transfected control vector cells (pCR3.1). However, during the stable cell line selection, constitutive overexpression of CRT resulted in cell spreading (see Supplemental Figure S1A at http://ajp.amjpathol.org) followed by cell death within 2 weeks. Therefore, an inducible-CRT bladder cancer cell line was generated by tetracycline-regulated gene system. Cells were treated with 1 μg/mL tetracycline for 48 hours to induce CRT mRNA and protein expressions (Figure 1,B and D). Cell morphology was shown in Figure 1A, and the shape factors calculated by MetaMorph software were not significantly different between pCR3.1 and CRT-RNAi or between #5y and #5yCRT cells (see Supplemental Figure S1B at http://ajp.amjpathol.org).

Cell Proliferation Rate Is Lower in CRT Knockdown Cells

To understand how CRT regulates cell behavior, we used functional assays to investigate the physiological effects of CRT expression in bladder cancer cells. Their cell cycle was analyzed by flow cytometry. In these assays, the CRT-RNAi group showed more cells arrested in the G1 phase (41%) than the control group (30%), which indicated that CRT knockdown suppressed cell proliferation (Figure 2A, left panel). Nevertheless, our results showed that there was no significant difference between #5y and #5yCRT (Figure 2A, right panel). Cell proliferation rate was also assessed by MTT assays over several time points after plating. The MTT readings for CRT-RNAi cells were significantly lower than the MTT readings from control cells (pCR3.1) at 24, 48, and 72 hours (Figure 2B, left panel). However, no significant difference was observed between CRT overexpressed cells

![Figure 1](http://ajp.amjpathol.org)  
**Figure 1.** J82 bladder cancer cells were stably transfected with CRT overexpressing and RNAi plasmids. **A:** Cell morphology of CRT in differentially expressed cell lines in serum-containing medium. #5y and #5yCRT cells were stimulated with 1 μg/mL tetracycline for 48 hours to induce CRT expression. Scale bar = 200 μm. **B:** mRNA expressions were confirmed by real-time PCR in CRT knockdown and overexpression stable cell lines. CRT mRNA expression was normalized to the internal control, GAPDH. **C:** Western blot analysis shows protein expression in CRT-RNAi stable cell lines. The human β-actin level was used as a loading control. **D:** An endogenous CRT protein band (~60 kDa) was both detected in #5y and #5yCRT cells. An exogenous YFP-CRT fusion protein (~87 kDa) was only detected in #5yCRT cells. All of the results are repeated in at least three independent experiments. Each bar of the histogram represents quantified results and are shown as the mean ± SD. Statistical differences were compared with the control level (*$P < 0.01$, **$P < 0.001$).

![Figure 2](http://ajp.amjpathol.org)  
**Figure 2.** Knockdown of CRT altered the proliferation rate of bladder cancer cells. **A:** The cell cycle was analyzed by flow cytometry. **Left:** CRT-RNAi cells showed proportionally more cells in the G1 phase than the S and G2/M phases compared with the vector control group (pCR3.1). **Right:** Cells that induced CRT expression did not promote cell cycle transit. **B:** Cell proliferation was determined by MTT assay. Cells (2500 cells/well) were seeded in 96-well plate. Cell numbers were determined by measuring the absorbance of 550 nm at 24, 48, and 72 hours after plating. **C:** Apoptosis was evaluated after starvation for 24 hours and stained with Annexin V. Treatment of actinomycin-D (act-D; 5 μg/mL) is used as positive control. The number showed the percentage of early apoptotic cells (lower right quadrant). These results were confirmed by at least three independent experiments ($P < 0.01$, **$P < 0.001$).
and vector-transfected cells (Figure 2B, right panel). To estimate whether the decreasing numbers in the CRT-RNAi group was because of apoptosis, we used Annexin V/pro-pidium iodide staining to determine the apoptotic cells. Actinomycin-D, which is a potent inducer for apoptosis in several cell lines, was used as positive control. As shown in Figure 2C, the percentage of Annexin V–positive cells were in both pCR3.1 and CRT-RNAi cells, which indicated that knockdown of CRT did not induce apoptosis in J82 cells. These results justified that down-regulation of CRT inhibited proliferation of bladder cancer cells rather than induction of apoptosis.

Alteration of CRT Levels Affects Cell Attachment and Migration

We also used cell migration and attachment assays to explore the effect of CRT levels in J82 cancer cells. Equal numbers of cells were seeded on a collagen-coated plate for 20 minutes. Results showed that the attachment ability of the cell was suppressed in CRT-RNAi cells compared with transfected vector control cells (Figure 3A, bars 1 and 2). On the contrary, CRT overexpression enhanced cell attachment significantly than vector control cells (Figure 3A, bars 3 and 4). To determine whether the effects of CRT are universal to bladder cancer, we used another bladder cancer cell line to perform our experiments. Down-regulation of CRT in T24 bladder cancer cells also suppressed cell attachment to type-I collagen (see Supplemental Figure S2 at http://ajp.amjpathol.org), suggesting that the effects of CRT we observed are not only specific to J82 cell line. Furthermore, with the use of the modified Boyden chamber method, J82 cells with low CRT expression had lower migration capacities than control cells (Figure 3B, bars 1 and 2), and cells with higher CRT expression showed an opposite effect (Figure 3B, bars 3 and 4). Consequently, these results showed a positive correlation between CRT expression levels and cell attachment and migration in bladder cancer cells.

Phosphorylation Levels of FAK and Paxillin Are Lower in CRT Knockdown Cells

Papp and others reported that altering CRT expression in fibroblasts can affect cell motility and adhesion through and vector-transfected cells (Figure 2B, right panel). To estimate whether the decreasing numbers in the CRT-RNAi group was because of apoptosis, we used Annexin V/pro-pidium iodide staining to determine the apoptotic cells. pCR3.1 and CRT-RNAi cells were starved for 24 hours. Actinomycin-D, which is a potent inducer for apoptosis in several cell lines, was used as positive control. As shown in Figure 2C, the percentage of Annexin V–positive cells were in both pCR3.1 and CRT-RNAi cells, which indicated that knockdown of CRT did not induce apoptosis in J82 cells. These results justified that down-regulation of CRT inhibited proliferation of bladder cancer cells rather than induction of apoptosis.

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Papp and others reported that altering CRT expression in fibroblasts can affect cell motility and adhesion through
regulating c-Src and FAK activity. Paxillin is one molecule of the focal adhesion complex which regulates the cell adhesion mechanism. On the basis of those previous studies, we used Western blotting to analyze the downstream signaling pathway affected by CRT in our system. Phosphorylation levels of Src (pY418), FAK (pY397), and paxillin (pY118) were also examined. As shown in Figure 4, the phosphorylation levels of FAK (−40%) and paxillin (−40%) decreased in CRT knockdown cells, whereas no difference in Src phosphorylation was observed.

Knockdown of CRT Inhibits Tumor Growth in Vivo

Stably transfected cells were subcutaneously injected into nude mice to determine the effects of CRT on tumor growth. Tumor volumes were measured every 3 days. Compared with tumors of control mice, tumors of mice injected with CRT-RNAi were significantly smaller after 21 days. The mean volume of tumors of CRT-RNAi clones was suppressed 60% more than that of control clones at day 35 (Figure 5A). Moreover, we further determined the end-stage apoptosis in the injection of cells from nude mice. Tumor sections from both groups were analyzed by TUNEL assays. The number of TUNEL-positive cells in the control group was not significantly increased in comparison to the CRT-RNAi group (Figure 5B). These results are consistent with our observations in vitro.

Knockdown of CRT Inhibits Metastasis of Bladder Cancer Cells

In addition, we further confirmed that the metastatic capacity was lower in the CRT-RNAi subcutaneous injection model. After sacrificing the mice, we found metastatic nodules in the liver of control mice but not in CRT-RNAi-injected mice (Figure 6A). These results strongly suggest that expression levels of CRT are closely related to the metastatic behavior of bladder cancer cells. To further confirm this hypothesis, we performed a tail vein injection

Figure 5. Knockdown of CRT diminishes tumor formation in nude mice. A: Mice were subcutaneously injected with vector control cells (closed circles) or CRT-RNAi cells (open circles), and tumor sizes were measured over time. After 20 days, tumor sizes significantly differed between the two groups. Knockdown of CRT suppressed tumorigenesis in bladder cancer cells. B: Determination of TUNEL-positive cells for injection of cells from mice (high power field, ×200). The arrows indicate the positive cells. No significant difference was observed between the two groups. Each bar of the histogram represents quantified results of the TUNEL-positive/field and is shown as the mean ± SD.

Figure 6. Knockdown of CRT inhibits metastasis of bladder cancer cells. A: Nude mice were subcutaneously injected with 10^7 cells and sacrificed after 45 days. There were obvious metastatic nodules in the liver of the control group (shown by arrows) but not in the CRT-RNAi group. B: Lung and liver metastases were performed by an intravenous injection. In total, 10^7 cells (control and CRT-RNAi) were injected into the tail vein of severe combined immunodeficient mice (n = 5 and n = 7, respectively). After 40 days, both groups had lung metastases, but the area of lung metastasis was higher in the control group. Liver metastasis was only observed in control mice.
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Table 1. Lung and Liver Metastatic Incidence in CRT-Stable Cell Lines

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Metastatic area is indicated by the following: +, <25%; ++, ≥25%; ++++, ≥40%.

Discussion

CRT is well established as a multifunctional protein that regulates cell behavior. In recent studies, it was shown that CRT is located in the cytosol and extracellular environments and on cell surfaces to modulate diverse physiological and pathologic effects. Many researchers reported that in some cancers CRT is up-regulated in tumor tissues more often than in normal tissues. Kageyama et al. identified that CRT levels are higher in urothelial tissues more often than in normal tissues. Kageyama et al. reported that in some cancers CRT is up-regulated in tumor biological and pathologic effects. Therefore, we conclude that vector control cells and 71% (5 of 7) of CRT-RNAi-injected mice developed lung metastasis. Although lung metastasis was observed in both groups, the percentage of metastatic areas was much higher in the control group (Figure 6B; see also Supplemental Figure S3 at http://ajp.amjpathol.org). From the two mice models, we can conclude that vector control cells induced tumor formation and visible liver and lung metastasis, whereas CRT-RNAi cells formed smaller tumors and fewer metastatic nodules in the liver and lungs. These results are summarized in Table 1.

There is evidence that CRT is colocalized with the α5β1 integrin via the N-domain, and surface CRT interacts with the collagen receptors, α5β1 integrin and glycoprotein VI, in human platelets. Furthermore, active integrins increase the association between CRT and α5β1 integrin. Abnormal cell migration is a typical characteristic of cancer metastasis, and integrins, an important modulator for cell adhesion and migration, are involved in this process. Although CRT is proposed to play a suppressive role in growth and metastasis of prostate cancer, it was also shown that mRNA and protein expressions of CRT are elevated in highly metastatic prostate cancer cell lines. This indicates a possible correlation between CRT and metastasis. According to these references, we hypothesized that our observation of down-regulated CRT diminishing cell migration, adhesion, and metastatic abilities in bladder cancer cells might have been because of the reduced interaction between CRT and integrins.
Protein tyrosine phosphorylation is a major event for the cell adhesive mechanism.\textsuperscript{43,44} Cell adhesiveness decreases in CRT-underexpressing mouse L fibroblasts that contain higher levels of active c-Src.\textsuperscript{12} However, the results of our experiments showed that c-Src activity is unaltered, which led us to think that the relationship between CRT and c-Src activation may differ, depending on the type of cell. Although there was no difference in c-Src phosphorylation, we observed decreases in phosphorylation levels of other crucial focal contact proteins such as FAK and paxillin. FAK is a cytoplasmic non-receptor tyrosine kinase identified as the downstream signaling molecule of integrin-dependent cell adhesion. It is also known that the c-terminal of FAK is the binding site for the integrin-mediated protein, paxillin.\textsuperscript{45} A previous study showed that breast cancer cells with high metastatic capacity have increased mRNA expression and paxillin protein levels.\textsuperscript{46} In addition, FAK was also proven to be a key signaling factor in tumor initiation, angiogenesis, and metastasis.\textsuperscript{47} This evidence supports our data which showed that decreased CRT expression in bladder cancer inhibits cancer metastasis through regulating FAK and paxillin phosphorylation.

CRT regulates various cell behaviors which suggests that it may also play a part in cancer development. Much clinical research has shown a correlation between CRT expression levels and clinicopathologic factors in different cancers. CRT is highly expressed in gastric tumor tissues and significantly correlated with tumor serosal invasion, lymph node metastasis, and microvesSEL density.\textsuperscript{21} Moreover, CRT is up-regulated in bladder tumor tissues, and the concentration of urinary CRT has a ten-dency to increase in histologic grade and pathologic T stage in bladder cancer.\textsuperscript{19,20} If a patient with bladder cancer is diagnosed with advanced-stage (T2 to T4) tumors, it means that the cancer cells possess a higher invasive ability to grow into the muscle layer and can even spread to areas outside the bladder which increases the incidence of tumor metastasis. These results provide strong evidence that supports the phenomenon of bladder cancer cells that express lower levels of CRT having diminished tumor growth and metastasis in mice models. Therefore, we concluded that CRT might play an important role in bladder cancer metastasis.

On the basis of the clinical data published by Kageyama et al.,\textsuperscript{19,20} our results further clarify how CRT modulates the physiological mechanisms in bladder cancer progression. These findings, for the first time, provide evidence that altered CRT expression affects bladder cancer tumorigenesis and metastasis. In the future, we will attempt to investigate detailed mechanisms of CRT-mediated adhesion in bladder cancer to optimize the research modality.

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References


