Increased expression of cortactin is associated with prostate cancer progression/invasiveness and metastasis

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Abstract: Metastasis is the major cause of prostate cancer deaths. Cortactin is a protein that has been shown to be critical for the cancer cell migration and invasion and play an important role in tumor metastasis. However, the clinical significance of cortactin in prostate cancer and the role of cortactin in the progression and metastasis of prostate cancer are still not clear. In this study, we examined the cortactin expression in benign prostate and different stages of prostate cancer tissues including 26 BPH, 82 untreated primary cancer, 58 NHT cancer, 20 CRPC, and 13 metastatic prostate cancer tissues, and analyzed its relationship with clinicopathological factors. The result showed that cortactin expression increased gradually in different stages of cancer progression and was associated with prostate cancer metastasis. Further functional analysis showed that knockdown of cortactin protein by siRNA led to a marked reduction in PC-3 cell invasive ability and extracellular matrix degradation. Our findings suggested that expression of cortactin is correlated with the progression of clinical prostate cancer to more aggressive stages and provided support for the role of cortactin in prostate cancer cell invasion. Cortactin may serve as a promising biomarker and therapeutic target for metastatic prostate cancer.

Keywords: Cortactin, prostate cancer, progression, metastasis

Introduction

With an estimated 233,000 newly diagnosed cases and 29,480 deaths within the United States in 2014, prostate cancer is the most common cancer (excluding basal and squamous cell skin cancers) as well as the second leading cause of cancer-related deaths in American males [1]. An important caveat is that Prostate cancer incidence and mortality in most native Asian populations have gradually increased [2]. Metastasis to the bone occurs in the majority of the cases as this disease progresses to its advanced stages, which results in high morbidity and mortality with an estimated 5-year survival rate of 25% and median survival of approximately 40 months [3]. Despite improvements in detection and treatment of prostate cancer at its early stages, many patients that succumb to the advanced stages of the disease still ultimately face death as the metastasized prostate cancer develops resistance to conventional treatments, such as hormonal ablation therapy [3, 4]. There are currently no effective means of treating or preventing the metastasis of prostate cancer, and improvements in therapy will be contingent upon a better understanding of the biology of this disease.

The remodeling and degradation of the extracellular matrix (ECM) is a crucial step of cancer metastasis. Invadopodia are actin-driven protrusions to drive cancer cell migration and invasion and play an important role in ECM degradation [5-7]. Cortactin, first identified as a major Src kinase substrate [8], is a protein found strongly localized to invadopodia and has been shown to be critical for its function [9]. Physiologically, cortactin binds to Arp2/3 complex and F-actin (filamentous actin) at its N-terminal acidic (NTA) and tandem repeats domains, respectively. At its C-terminal end also has a proline-rich domain that contains phosphorylation sites for a number of kinases, as well as a SH3 domain that contains binding
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sites for a variety cytoskeletal and membrane trafficking proteins such as N-WASP, dynamin and WIP [10]. As such, cortactin is thought to function as a regulator of branched actin assembly, as well as a scaffolding protein that regulates cytoskeletal arrangement and membrane trafficking. There is also speculation that the primary role of cortactin in invadopodia function is to promote protease secretion responsible for the degradation of ECM. Frequently found to be overexpressed in a variety of cancers as a result of chromosome 11q13 amplification [11], the overexpression of this protein is known to increase tumor aggressiveness, possibly via promoting tumor invasiveness and metastasis [10, 12-15].

Despite our current understanding, however, the study of cortactin in prostate cancer is very limited and the role of cortactin in the progression and metastasis of prostate cancer is still not clear. In our present study, we determined cortactin expression in different stages of clinical prostate cancer tissues, including localized hormone naïve prostate cancer, castration resistant prostate cancer (CRPC) and metastatic prostate cancers; further functional studies showed the effect of cortactin knockdown on cell migration and invasion in the PC-3 prostate cancer cell line. Finally, we demonstrated that cortactin may be essential for the formation of invadopodia that facilitate invasiveness in PC-3 cells.

Materials and methods

Materials

Chemicals, stains, solvents and solutions were obtained from Sigma-Aldrich Canada Ltd, Oakville, ON, unless otherwise indicated.

Clinical prostate cancer tissues

The 194 prostate cancer specimens were obtained from The Second Hospital of Tianjin Medical University. Specimens were obtained from patients, with their informed consent, following a protocol. The study was approved by the research ethics committee of Tianjin medical university. Tissue microarrays (TMAs) were constructed. The H&E slides were reviewed and the desired areas were marked on them and their correspondent paraffin blocks. All the specimen were from radical prostatectomy except castration resistant prostate cancer (CRPC) samples that obtained from transurethral resection of prostate (TURP). In addition, sections from 8 lymph node, 2 lung and 3 bone metastatic prostate cancers were obtained for cortactin protein analysis.

Histopathological and immunohistochemical staining

Preparation of paraffin-embedded tissue sections were carried out as previously described. Immunohistochemical staining was conducted by Ventana autostainer model Discover XT™ (Ventana Medical System, Tuscan, Arizona) with enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit by using mouse monoclonal anti-cortactin antibody (clone 4F11, Millipore, Billerica, MA).

Cortactin scoring

Cytoplasmic cortactin protein staining in tissue samples was evaluated by two independent pathologists in blinded analyses. Specimens were graded from 0 to 3+ in intensity to represent a range from no staining to strong staining. The analyses were performed on the mean value of cortactin protein expression for each specimen.

Cell cultures

The human prostate cancer cell lines, PC-3, were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 medium (Stem Cell Technologies, Vancouver, BC), supplemented with 10% fetal bovine serum (Gibco-BRL, Burlington, ON), in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Subculturing was carried out using standard techniques, including trypsinization using 0.25% trypsin/1.0 mM EDTA.

Small interfering RNA (siRNA) and cell transfection

siRNAs targeting cortactin band negative control (scrambled) siRNA were purchased from Qiagen. The cortactin-targeting RNA were siRNA 1,5'-CCAGGAGCAUAUCAACAUATT-3' and 5'-UAUGUUGAUAUGCUCCUGG-3', and siRNA 2,5'-GCAACUUAUUGAUCUGAATT-3' and 5'-UUUCAGAUCAUAUAGUUGCAT-3'. Vehicle and scrambled siRNA were used as controls. To examine the effect of the siRNAs on cortactin expression,
PC-3 cells were plated in 6-well plates in antibiotic-free RPMI-1640. After 20 hours, the cells were transfected with 40 nmol/L siRNA in Lipofectamine™ 2000 reagent following the manufacturer's instructions. Vehicle (Lipofectamine™ 2000) and scrambled siRNA were applied in separate wells. Following 48 hours incubation, the PC-3 cells were harvested for western blot analysis, migration assay and matrigel invasion assays.

**Western blotting**

Cells growing on a 6-well plate were harvested by scraper and protein lysates were prepared using modified RIPA buffer (1% NP-40, 0.5% sodium deoxycholic acid) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Total lysate protein was determined using the BCA protein assay (Pierce, Rockford, IL). Typically, 5 µg of proteins were separated by electrophoresis through 7.5% SDS polyacrylamide gels and transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA). Membranes were incubated with mouse anti-cortactin monoclonal primary antibody (clone 4F11, Millipore, Billerica, MA) or rabbit anti-actin polyclonal primary antibody (Sigma) for 1 hour at room temperature. After washing and incubation with secondary antibodies, bands were visualized on autoradiography film by ECL (Super Signal West Femto Maximum Sensitivity Substrate, Pierce).

**Matrigel invasion assays**

Assays were performed using modified Boyden chambers consisting of 8 µm pore filter inserts in 24-well plates (BD Biosciences, San Jose, CA). Cells in serum-free RPMI-1640 medium were plated (1×10⁵/well) on Matrigel-coated of the upper compartments and incubated at 37°C in a CO₂ incubator, using RPMI-1640 containing 5% FBS in the lower chambers as a chemoattractant. After 22 hours the inserts were pulled out and the remaining, non-invading cells on the upper surface removed with a cotton swab. The cells on the lower surface of the membrane were fixed in methanol, air-dried and stained with 0.1% crystal violet for 10 minutes. The cells on each membrane were counted in five fields (at 100× magnification) using a light microscope. Data are presented as percentages calculated by normalizing the values obtained for the untreated cells as 100%.

**Fluorescent gelatin degradation assay**

Cross-linked-Alexa 568-conjugated gelatin matrix-coated coverslips (Molecular Probes, Eugene, OR) were prepared following manufacturer's protocol. Gelatin-coated coverslips were quenched with RPMI containing 10% fetal bovine serum at 37°C for 60 min prior to plating cells. To assess degradation of Alexa 568-gelatin matrix, PC-3 cells were cultured on Alexa 568-gelatin-coated coverslips for 16 h. Cells were fixed and stained for actin with Alexa 488-phalloidin (Invitrogen). Gelatin matrix and actin-stained cells were viewed and photographed with a Nikon confocal laser-scanning microscope. Images were saved in TIF image format and processed by using Photoshop.

**Statistical analysis**

All values presented are expressed as the means ± S.E. of three or more experiments done at different times normalized to intraexperimental control values. Mann-Whitney U-test was used to determine if a significant difference of cortactin expression present between groups. Statistical significance in this study was set as P≤0.05.

**Results**

**Expression of cortactin protein in clinical prostate samples**

Three tissue microarrays (TMAs) of clinical prostate samples, including 26 BPH, 82 untreated primary cancer tissues, 58 NHT cancer tissues, 8 recurrent cancer tissues, 20 CRPC tissues, and additional 13 metastatic prostate cancer tissues were examined for cortactin protein expression. Preliminary visual scoring suggested that cortactin immunoex-
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Expression in prostate cancer was higher than that in benign prostatic hyperplasia. The stained tissue shows cytoplasmic staining in the cancer cells. Smooth muscle cells were uniformly scored positive for cortactin expression. Result of quantitative analysis of cortactin staining is provided in Table 1, and representative images are shown in Figure 1. These data were obtained from staining scores of from 207 prostate cancer specimens. Overall, cortactin expression was increased in prostate cancer tissues compared to benign prostate. The

Figure 1. Representative cortactin immunostaining in benign and malignant prostate tissues. A: Negative to weak expression of cortactin in benign prostate tissue; B: Weak cortactin expression in primary untreated prostate cancer tissue; C, D: Moderate cortactin expression in NHT and recurrent prostate cancer; E: Strong cortactin expression in CRPC sample; F: Strong cortactin expression in lymph node metastasis sample. (Original magnification ×200).
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**Table 2. Significance of differences in cortactin staining scores according to tissue type**

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>BPH</th>
<th>Untreated</th>
<th>NHT</th>
<th>Recurrent</th>
<th>CRPC</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHT</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrent</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.075</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRPC</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.286</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Significances are expressed as P value derived from multiple comparisons as described in statistical methods.

Staining of cortactin increased gradually in different stages of cancer progression. Metastatic prostate cancer tissue showed highest cortactin expression in the cohort. The significance of differences in cortactin staining scores according to different prostate tissues was shown in Table 2.

**Knockdown of cortactin protein by siRNA decreases PC-3 cell invasive ability**

In vitro cell invasion assays were used to examine the effect of reduced cortactin protein expression on migration and tissue invasion of PC-3 cells. Reduction of cortactin expression in PC-3 cells was obtained by transfection of the cells with cortactin-targeting siRNAs. As shown by Western blot analysis, cortactin protein expression was markedly reduced 48 h after transfection with siRNA1 and 2, in contrast to cells transfected with the vehicle (lipofectamine™ 2000) or scramble siRNA (Figure 2A). In the cell invasion assay, no significant difference was observed between the scramble siRNA-treated and vector-treated cells (P=0.27). siRNA-induced reduction of cortactin expression resulted in a markedly reduction of invading cells as compared to vector-treated cells (25.9±5.9% and 15.4±5.3% in siRNA1- and siRNA2-treated cells, respectively; P=0.01 and P<0.01 respectively) (Figure 2B). These studies demonstrate that the cortactin gene has an important function in the tissue invasive properties of PC-3 cells.

**Knockdown of cortactin protein by siRNA decreases extracellular matrix degradation of PC-3 cells**

To investigate the role of cortactin in prostate cell invasion and ECM degradation, PC-3 cells incubated with cortactin-targeting siRNA and negative control siRNA for 48 h were plated on Alexa 568-conjugated gelatin matrix for 16 h. Cells were fixed and stained for actin with Alexa 488-phalloidin. PC-3 cells treated with negative control siRNA displayed areas of actin enrichment in the cell peripheries as well as within the cell body (Figure 3A). Apparent gelatin degradation was present underneath the cell body (Figure 3B). Degradation was confined within the cell boundary. Actin-enriched invadopodia-like structures was overlapping with focal degradation of gelatin matrix (Figure 3C). Such ECM degradation was significantly reduced in cortactin knock-down PC-3 cells (Figure 3D-F).

**Discussion**

Overexpression of cortactin, an actin-nucleation-promoting factor, has been linked to various types of invasive cancers, including head and neck squamous cell carcinoma (HNSCC) [12, 13, 16, 17], lung cancer [18], breast cancer [19, 20], colorectal cancer [21], oral cancer [22], melanoma [23] and brain gliosarcoma [24]. However, the role of cortactin in prostate cancer is still not clear. To determine the correlation between cortactin expression and the progression of prostate cancer, we examined the expression of cortactin in a clinical prostate cancer cohort. As shown in Figure 1 and Tables 1 and 2, immunohistochemical staining has revealed an increased cortactin expression in malignant versus benign prostate tissues. Consistent with the knowledge that cortactin is frequently overexpressed in advanced, invasive stages of a variety of other cancers and that its overexpression increases tumor aggressiveness [25-28], our results also showed an increase in expression in the CRPC/metastatic prostate cancer tissue samples compared with the recurrent/NHT samples. This suggests a positive correlation between cortactin expression and the progression of prostate cancers. It also suggested that cortactin could be used as a marker for aggressive prostate cancer.

To further study the function of cortactin in prostate cancer, cortactin expression was knocked down by siRNA in highly invasive PC-3 cells. The decrease of cortactin expression
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resulted in a significantly lower number of invaded cells in Matrigel invasion assay compared to vehicle control and non-targeting siRNA. This result is consistent with previous findings in DU145 cells. Interestingly, Nakane et al. have reported cortactin expression to be higher in DU145 cells compared to PC3 cells, in the latter of which cortactin expression was not elevated. Since PC3 cells have been identified to possess a higher capacity to invade the extracellular matrix and therefore a higher metastatic potential compared to DU-145 cells [29], this may imply that the invasiveness of prostate cancer is not dependent (or is dependent to a relatively low extent) on the level of cortactin expression itself, but more on other factors stemming from cell type and physiology.

It has been reported that cortactin localizes strongly to invadopodia, and that it may play an important role in the localization and secretion of matrix metalloproteases (MMP) to those structures during ECM degradation [25, 26, 31]. To understand whether cortactin may mediate formation and/or functions of the similar structures in the invasive prostate cancer, we examined the ability of PC3 cells to degrade the ECM using a fluorescent gelatin degradation assay. Classically,
invadopodia are identified by observing the colocalization of F-actin puncta to individual sites of matrix degradation [32]. As can be seen in the negative control PC3 cells expressing native levels of cortactin in Figure 3A-C, puncta formed by actin congregations directly overlapped with the dark areas of degraded gelatin that are indicative of invadopodia-like structures. Neither sites of degradation nor sites of actin congregation were observed in the case of cortactin knockdown (Figure 3D-F), suggesting that cortactin may be critical for the formation of invadopodia-like structures. This observation challenges the idea put forth by Nakane et al. that the contribution of cortactin in the formation of invadopodia is relatively small, as no morphological changes have been observed in their case despite cortactin knockdown [33]. However, it does not rule out the possibility that these differences observed may be due to differences in PC3 and DU145 physiology. At this point, precise mechanisms by which cortactin contributed to the formation of invadopodia in PC3 cells remains unknown.

Drawing upon previous speculations, it is possible that cortactin, being a relatively weak activator of the Arp2/3 complex for branched actin nucleation, may function primarily to stabilize branched actin only after they have been formed [26]. It is also unknown whether cortactin may have contributed via direct interaction with F-actin, or via mediating degradation in which case degradation products generated from MMP activity may promote new invadopodia formation by way of a feedback loop [34].

Conclusions

Overall, this study has provided evidences that cortactin may be correlated with the progression of clinical prostate cancer to more aggressive stages. Our findings have also provided support for the role of cortactin in prostate cancer cell invasion, and the formation of invadopodia-like structures in PC3 cells. Cortactin may serve as a promising biomarker and therapeutic target for metastatic prostate cancer.

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Disclosure of conflict of interest

None.

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