Ninjurin1 Is Up-regulated in Circulating Prostate Tumor Cells and Plays a Critical Role in Prostate Cancer Cell Motility

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Abstract. Background/Aim: Ninjurin1 is a 17-kDa membrane protein that is highly expressed in circulating tumor cells (CTCs) obtained from locally-advanced prostate cancer patients. As CTCs are implicated in the initiation of distant metastasis, we examined the potential contribution of Ninjurin1 to the motility of prostate cancer cells. Materials and Methods: Ninjurin1 expression was evaluated in CTCs harvested from seven locally advanced patients with no metastatic hallmarks using real-time polymerase chain reaction (PCR). The role of Ninjurin1 in cell motility was investigated using small interfering RNA (siRNA), neutralizing antibodies against Ninjurin1 and Ninjurin1-overexpressing adenoviruses. Results: Ninjurin1 was ranked as the most significantly up-regulated adhesion protein identified by RNA-Seq analysis. Both Ninjurin1 down-regulation by siRNA and neutralizing antibodies blocking Ninjurin1 homophilic interactions effectively inhibited cell motility. In contrast, cell motility was enhanced in prostate cancer cells infected with adenovirus enabling Ninjurin1 overexpression. Conclusion: Ninjurin1-neutralizing antibodies or Ninjurin1-targeting siRNA merit further development for patients with metastatic potential.

Prostate cancer is the second most common cancer among males in Western countries (1). The disease progresses from high-grade prostate intra-epithelial neoplasia to locally invasive carcinoma and, ultimately, to metastatic cancer, the latter of which is associated with a high mortality rate. Metastases to the bone, lymph nodes, liver and lung account for more than 10% of cancer-related deaths in men. Patients with prostate cancer have a relatively favorable 5-year survival rate, partly due to early detection and effective androgen deprivation therapy. However, a significant number of prostate cancer patients become resistant to this type of treatment. Patients who develop androgen-independent and metastatic prostate cancer have few therapeutic options, with the 5-year survival rate, associated with these patients, being as low as 12.6%. Therefore, it is imperative to investigate the mechanisms underlying prostate cancer metastasis to facilitate the discovery of novel therapeutic targets and treatment approaches. As circulating tumor cells (CTCs) include precursors that are able to colonize distant metastatic sites, they are likely to provide insight into the genomic features associated with metastatic tumor cells.

Capturing sufficient amounts of the extremely limited number of CTCs in the blood, required for sequencing, is an enormous challenge. To date, the most effective strategy for purifying CTCs is based on the utilization of membrane surface proteins expressed on CTCs (2). Using a microfluidic capture platform coated with EpiCAM-reactive antibodies, several studies have been able to obtain CTCs and analyze their genetic profile. Allele-specific polymerase chain reaction (PCR) amplification, array comparative genomic hybridization (CGH) and next-generation sequencing, as well as whole-genome amplification have revealed somatic single nucleotide variants and copy number variations associated with prostate cancer (3-5). Furthermore, single molecule RNA sequencing has demonstrated that the wingless-related integration site (WNT) pathway is critical to the suppression of anoikis and to the enhancement of anchorage-independent sphere formation and metastatic potential (2). Analyzing genomic mutations in CTCs using established methods for cell enrichment and isolation, genomic amplification, library qualification and census-based sequencing has become a standard protocol for evaluating prostate cancer (6). We developed an alternative approach in which a replication-
Competent adenovirus is introduced exclusively into prostate-specific antigen/prostate-specific membrane antigen (PSA/PSMA)(+) cells, with this method not being dependent on EpiCAM or specific membrane proteins (7). Interestingly, this approach enabled us to detect circulating prostate cancer cells from several patients who had not been clinically diagnosed with metastatic tumor nodules. In addition, RNA-Seq analyses demonstrated that several metastasis-associated genes were up-regulated in these patients and Ninjurin1 was top-ranked among most significantly changing adhesion molecules.

Ninjurin1 (nerve injury-induced protein 1), a 17-kDa homophilic molecule that localizes to the cell membrane, is strongly induced by nerve injury in dorsal root ganglion neurons and Schwann cells (8). Thus, the myeloid cells of patients with experimental allergic encephalomyelitis or active multiple sclerosis express high levels of Ninjurin1, a phenomenon that facilitates the infiltration of inflammatory myeloid cells into the central nervous system (9). Another study reported that Ninjurin1 is up-regulated in highly migratory T cells in the lungs of an experimental autoimmune encephalomyelitis (EAE) rat model and plays an important role in invading central nervous system vessels (10). In addition to its association with nervous system pathologies, Ninjurin1 is over-expressed in multiple cancers, including hepatocellular carcinoma (11), acute lymphoblastic B-cell leukemia (12) and high-grade bladder cancer (13). Furthermore, we observed enhanced Ninjurin1 expression in docetaxel-resistant prostate cancer cells. As little is known regarding the metastatic-related function of Ninjurin1 in cancer cells, we investigated how it would contribute to motility of CTCs during prostate cancer progression.

**Materials and Methods**

**Cells.** The human prostate cancer cell lines PC3 and DU145 (ATCC, Manassas, VA, USA) were maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Docetaxel-resistant prostate cancer cells were established by prolonged exposure to 5 nM docetaxel. CTCs were maintained in Dulbecco’s high glucose MEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics.

**Counting CTCs in prostate cancer blood using Ad5/35E1aPSESE4.** For the separation of peripheral blood mononuclear cells (PBMCs) from whole blood, 5 ml of whole blood in a K2 EDTA tube was added to a 50 ml conical tube containing 4 ml of Ficoll-Paque PLUS (GE Healthcare Life Science, Pittsburg, PA, USA) and was gently mixed with PBS to reach a total volume of 10 ml. The PBMC sample was prepared in a 12-well plate as described (7). Then, PBMCs were infected with 0.01 multiplicity of infection (MOI) of Ad5/35E1aPSESE4 and determined whether to contain CTCs mediating green fluorescent protein (GFP) expression. Tumor cells were maintained in Dulbecco’s High Glucose MEM medium supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics.

**RNA-Seq analysis.** The control and test RNA, libraries were constructed using the SENSE mRNA-Seq Library Prep Kit (Lexogen, Inc., Vienna, Austria) following the manufacturer’s instructions with minor modifications. Briefly, 2 μg of total RNA from each sample was incubated with magnetic beads coated with oligo(dT) and all other RNA except for mRNA was removed with washing solution. The library was generated by randomly hybridizing starter/stopper heterodimers to poly(A) RNA bound to the magnetic beads. The starter/stopper heterodimers contained Illumina-compatible linker sequences. Reverse transcription and ligation reactions in a single tube extended the starter to the next hybridized heterodimer and the newly-synthesized cDNA insert was then ligated to the stopper. Second strand synthesis was performed to release the library from the beads and the library was subsequently amplified. Barcodes were introduced during the amplification of the library. High-throughput sequencing was performed with paired-end 100 sequencing using the HiSeq 2000 platform (Illumina, Inc., Sang Diego, CA, USA). RNA-Seq reads were mapped using the TopHat software tool (http://bioinformatics.oxfordjournals.org/content/25/9/1105.abstract) to generate the alignment file. The alignment file was used to assemble the transcripts, estimate the levels of each transcript and identify differentially expressed genes or isoforms using Cufflinks.

To identify novel targets of prostate cancer in CTCs, we carried out a robust cluster analysis and statistically analyzed the association between the RNA-Seq data from 2 CTC samples and the publically available RNA-Seq data from 3 prostate normal tissue samples. First, 153 genes associated with cell adhesion function were classified using the AmiGO database (14). We selected 56 genes with a ≥2-fold change in expression and ≥2 standard deviations between the CTC and normal tissue samples. Student’s t-test was used to evaluate differentially expressed genes with a cut-off p-value <0.05.

**PCR and real-time PCR analysis.** Total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer’s protocols. cDNA was synthesized from the extracted RNA in a total reaction volume of 20 μl containing 10 mM Tris-HCl, (pH 8.8), 50 mM KCl, 5 mM MgCl2, 1 mM each dNTP, 0.5 μg oligo(dT) primers, 25 U RNase inhibitor and 15 U reverse transcriptase (Invitrogen) for 5 min at 42˚C and, then, for 5 min at 95˚C. The following primers were used in the PCR reactions: Ninjurin1 in cancer cells (5’-TGA GGG CCG TAG ACC TTG TA-3’ and 5’-TAG AAA AGT GGG TGC TGG GA-3’), Ninjurin1 in CTC (5’-TGG TCC TCA TCT CCA CCT CC-3’ and 5’-CAT GTC CAT CAA GGG CTT CT-3’), GAPDH (5’-TGG TCA CCA GGG CGT CT-3’ and 5’-TCC GGG AAG ATG ATG GGA -3’) and HPRT1 (5’-AGA CTT TGG TTC CCT TGG GCA-3’ and 5’-TCA AGG GCA TAT CTC ACA ACA A-3’). The PCR reaction was conducted using the following conditions: a 10-min preheating step at 95˚C, 30 cycles of 30 s at 95˚C, 3 s at 60˚C and 30 s at 72˚C, and 10 min at 72˚C. For real-time PCR analysis, the same primers probed with fluorescence were added to each reaction mixture and the reactions were conducted using a real-time PCR instrument (Roche, Indianapolis, IN, USA).

**Invasion assay.** Cells (1x10⁴ cells per well) were seeded into the 96-well plate provided with the Oris™ Cell Invasion Assay Kit (Platypus, Madison, WI, USA). The plate was incubated for 48 h at 37˚C and the stoppers were then removed. Collagen I overlay was added to the wells to create a 3-D extracellular matrix (ECM) environment for invasion and the cells were, then, incubated for 1 h.
at 37°C. After cell culture medium was added, the cells were allowed to invade for 72 h. The membranes were stained with 4',6-
diamidino-2-phenylindole (DAPI) before images were captured. The
sequences of the sense and antisense strands of the small interfering
RNAs (siRNAs) targeting Ninjurin1 are as follows: *Ninjurin1* siRNA
(sense 5'-GUG UUC AUC UAC GUG GUA G-3' and antisense 5'-
C UACCACGACGUA GAAGCAC-3'). The AccuTarget™
Negative Control siRNA (Bioneer, Daejeon, South Korea) was used as
a negative control. siRNA was custom synthesized and the
negative control siRNA was purchased from Bioneer (Daejeon,
Republic of Korea). For the siRNA experiments, 2x10^5 cells were
seeded in each 60-mm culture dish. Once cells had grown to 70%
confluence, a mixture of 30 μl of siRNA (20 pmoles/μl) and 30 μl
of Lipofectamine 2000 reagent (Invitrogen) was added to the
medium of each dish. After 48 h, cells were harvested for the
invasion and migration assays.

**Western blot.** For the Western blot analyses, cells were lysed in
radioimmunoprecipitation assay (RIPA) buffer supplemented with
protease inhibitors (Sigma-Aldrich, St. Lois, MO, USA). Proteins
(20-50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide
gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene
difluoride (PVDF) membrane. The membrane was probed with
primary antibodies against Ninjurin1 (Cell Signaling, Danvers, MA,
USA) and β-actin (Sigma-Aldrich) and, subsequently, probed with
secondary antibodies (Jackson Immuno Research Laboratories, West
Grove, MA, USA). Peroxidase-conjugated secondary antibodies
were visualized using an Enhanced Chemiluminescence (ECL) Plus
kit (Thermo Fisher Scientific, Waltham, MA, USA).

**Results**

The expression levels of adhesion molecules are enhanced in
prostate CTCs. As previously reported (7), we developed a
replication-competent adenovirus to fluorescently label
CTCs derived from the blood of prostate cancer patients. As
shown in Figure 1A-B, PSA/PSMA(+) prostate cancer cells
in blood were labeled with fluorescence following infection with
0.01 MOI of Ad5/35E1aPSESE4. Subsequently, 39
leukocyte mixtures prepared from locally advanced prostate
cancer patients were examined. Blood from prostate cancer
patient at the metastatic stage was included as a positive
control. Interestingly, ten patients, who had local tumors but
had not been diagnosed with distant metastases, showed
more than one CTC as summarized in Figure 1C. Epithelium-derived tumor cells initiate cancer progression
and metastasis by undergoing a series of molecular events
associated with cell adhesion, including the down-regulation
of E-cadherin to reduce cell-cell adhesion and the up-
regulation of other cell adhesion molecules (CAMs). To
identify the CAMs that play key roles during migration and
invasion, we evaluated CTCs obtained from three patients
using RNA-Seq analysis. As shown in Figure 2A, 56 of the
153 genes associated with cell adhesion function identified in
the AmiGO database exhibited a ≥2-fold change in
expression with ≥2 standard deviations between CTC
samples and normal tissues. Ninjurin1 was the most
significantly differentially expressed gene (p=0.00426)
according to hierarchical gene clustering, suggesting that it
could play an important role in CTC motility in the blood.
To confirm the up-regulation of Ninjurin1 in CTCs from locally
advanced prostate tumor, seven different CTCs were cultured
and their total RNAs were tested for Ninjurin1 expression
using real-time PCR. Six out of seven patients exhibited
increased levels of *Ninjurin1* mRNA transcripts compared to
normal prostate samples (Figure 2B). Since the presence of
CTCs is indicative of potential distant metastases with poor
survival rates (15), Ninjurin1 overexpression in CTCs
suggests its critical role for cell motility.

**Inhibiting Ninjurin1 expression or activity reduces cell
motility in vitro.** As Ninjurin1 plays a critical role in the
motility of immune cells, we evaluated cell invasion and
migration in metastatic prostate cancer cells. To determine
the role of Ninjurin1 in metastatic prostate cancer cell
motility, we reduced *Ninjurin1* expression using siRNA.
*Ninjurin1* transcript levels were effectively reduced in PC3
(Figure 3A) and DU145 (Figure 3B) transfected with siRNA,
as confirmed by RT-PCR. Forty eight hours following the
transfection of 1x10^5 cells, they were incubated on a
transwell membrane for 48 h. As shown in Figure 3A-B,
depletion of *Ninjurin1* transcripts led to reduced motility as
assessed by invasiveness and migration assays. The
migration ability of DU145 was reduced with no statistical
significance. These results indicated that Ninjurin1 plays an
important role in cell motility. Next, we evaluated cell
invasion in cells treated with 500 ng/ml anti-Ninjurin1
antibodies to block Ninjurin1 activity. As shown in Figure 4,
the neutralizing antibody effectively blocked cell invasion
with statistical significance. Therefore, Ninjurin1 appears to
be a motility-associated protein that is required for CTCs to
enter the circulation.

**Ninjurin1 overexpression enhances cell motility in vitro.** To
evaluate the effects of Ninjurin1 overexpression, we
analyzed cell migration and invasion in cells infected with
an adenovirus expressing the *Ninjurin1* gene under the
cytomegalovirus (CMV) promoter (Figure 5). In contrast
with Ninjurin1 down-regulation, Ninjurin1 overexpression
strongly enhanced invasiveness and migration. These results
further confirmed that Ninjurin1 plays a role in prostate
cancer cell motility.

**Discussion**

Prostate cancer is a common cancer among men worldwide.
Prostate cancer progression is dependent on signaling
between androgens and androgen receptors; therefore, it is
conventionally treated using androgen-deprivation therapy.
However, most patients ultimately experience recurrent
Figure 1. Circulating tumor cells (CTCs) from locally advanced prostate cancer patients were marked with replication-competent adenovirus. A: Peripheral blood mononuclear cells (PBMCs) obtained from Ficoll isolation of 5 ml of blood were washed with PBS and re-suspended in 1 ml of DMEM supplemented with 10% FBS and 1% antibiotics. PBMCs were infected with 0.01 M Ad5/35E1aPSESE4. B: Post 48 h infection, cells were observed under a fluorescence microscope. Blood from bone-metastasized was included as a positive control. C: PBMCs prepared from thirty nine patients who were diagnosed of locally advanced prostate cancer were tested for CTCs upon infection with Ad5/35E1aPSESE4. One prostate cancer from metastasis was included as a positive control.
disease and progress to castration-resistant prostate cancer (CRPC). While CRPC patients with metastasis have therapeutic options for slowing cancer growth, metastatic CRPC remains lethal (16). Prostate cancer metastasis involves multiple steps, including angiogenesis, local migration, invasion, intravasation, circulation, extravasation, angiogenesis and colonization in distant sites (17). To facilitate the development of therapies targeting metastasis-associated molecules, it is imperative to determine which molecules are involved in the individual steps leading to bone metastasis. The observation that tumor-initiating cells with stem cell–like features and cells that have undergone epithelial-to-mesenchymal transition (EMT) can be found in CTCs indicates that CTCs may provide insight into molecules with the potential to address unmet needs in prostate cancer treatment. CTCs are likely to express various CAMs that mediate metastatic processes, such as vascularization, adherence to distant organs, extravasation, angiogenesis and proliferation. Therefore, this study focused on adhesion molecules expressed on the membrane of CTCs to identify and characterize factors that mediate metastasis. According to RNA-Seq analyses, Ninjurin1 was the most significantly up-regulated adhesion molecule expressed on CTC membranes compared with normal prostate epithelial cells. Bone marrow-derived macrophages depleted of Ninjurin1 exhibited a decrease in membrane protrusions and impaired cell motility. In Raw264.7 cells, overexpression of Ninjurin1 increased filopodial protrusion formation by activating
Figure 3. Down-regulation of Ninjurin1 transcripts reduces the migration of castration-resistant prostate cancer (CRPC) cells. siRNA targeting Ninjurin1 or control siRNA was transiently transfected into PC3 (A) or DU145 cells (B) and the effect of siRNA knockdown on Ninjurin1 expression was assessed using RT-PCR. The HPRT1 gene was used as a control. Cells ($1 \times 10^5$) were transfected with siRNA for 48 h and then transferred to a membrane for the invasion and migration assays. After 48 h, cells on the opposite side of the membrane were stained with hematoxylin and counted under a microscope.
Rac1, thereby promoting cell motility (18). Consistent with these findings, we found that blocking the homophilic interactions of membrane-bound Ninjurin1 in CRPC cells impaired invasion and migration, indicating that Ninjurin1 may play a critical role in cancer cell invasion and migration in the blood.

The bone marrow (BM) is a common site of prostate cancer metastasis and the detection of tumor cells in the BM at the time of diagnosis is associated with an increased risk of tumor recurrence (19). In addition, a previous study detected more disseminated tumor cells in the BM compared to CTCs obtained from blood samples (20). The finding that disseminated tumor cells in the BM further disseminate to other sites suggests that the level of disseminated tumor cells in the BM may be a better diagnostic and prognostic biomarker in prostate cancer (15). However, this observation may be a consequence of the sensitivity of the assays used to detect CTCs. Recent technological advances have enabled the detection, isolation, capture and characterization of CTCs from blood samples obtained in routine clinical practice. The CellSearch system (Veridex LLC, Raritan, NJ, USA) established a protocol utilizing antibodies against epithelial cell adhesion molecule (EpCAM), a molecule that is commonly expressed on the majority of primary and metastatic tumor cells but that may be down-regulated or absent in a subset of CTCs. Similarly, cells with stem cell-like features also lack EpCAM and cytokeratin expression. Thus, the identification of novel markers specifically expressed on CTCs that are not down-regulated during EMT may increase the potential for utilizing CTCs in the clinical setting. To overcome the limitations associated with the high level of sensitivity required to isolate CTCs, we developed a novel technology using a replication-competent adenovirus, Ad5/5E1aPSESE4, to label PSA/PSMA(+) cells (7). Interestingly, this adenovirus-based diagnostic tool provided similar results as conventional image-guided or PSA-based predictions of disease progression in most patients, although several patients with local tumors harbored too few CTCs in the blood to facilitate this type of analysis. These results suggest that CTC and disseminated tumor cells in the BM may have significant prognostic potential depending on the development of sensitive methods to detect CTCs.

Ninjurin1 is associated with cancer predisposition syndrome multiple self-healing squamous epitheliomata (21), a syndrome that renders patients vulnerable to multiple squamous cell skin cancers (22). With respect to hematological cancers, Ninjurin1 expression is elevated in acute lymphocytic leukemia. In addition, Ninjurin1 was identified by cDNA microarrays as a marker of B-lineage acute lymphoblastic leukemia (12). In solid cancers, Ninjurin1 was initially observed to be up-regulated in hepatocellular carcinoma associated with viral infection or liver cirrhosis (11). In non-muscle invasive bladder cancer, Ninjurin1 expression was associated with tumor progression, as demonstrated by immunohistochemical staining and tissue microarray (13). The cDNA microarray results demonstrated that Ninjurin1 expression was associated with non-muscle invasive bladder cancer recurrence (23).

Of note, a microarray-based genetic screen revealed that the tumor suppressor protein p53 enhances Ninjurin1 expression; this finding has resulted in an increased interest in the potential role of Ninjurin1 in carcinogenesis (24). In prostate cancer, some studies have demonstrated an association between p53 nuclear accumulation and poor differentiation, disease progression, metastasis and androgen-independent growth (25-27). Ninjurin1 expression appears to be regulated by p53 (28) and is associated with chemotherapy sensitivity.

In summary, Ninjurin1 expression was highly up-regulated in circulating tumor cells from some locally advanced prostate cancer and the inhibition of Ninjurin1 expression or molecular action greatly reduced prostate cancer cell motility. Therefore, inhibiting Ninjurin1 homophilic interactions using Ninjurin1-neutralizing antibodies or inhibitory peptides or down-regulating Ninjurin1-expression using siRNA targeting Ninjurin1 may contribute to novel therapeutic approaches that can provide survival benefits to patients with metastatic potential.

Conflicts of Interest

The Authors declare no conflicts of interest.
Figure 5. Overexpression of Ninjurin1 by adenovirus Ad5CMV.NINJ1 infection enhanced cell motility in metastatic prostate cancer cells. PC3 (A) or DU145 cells (1 × 10^5) (B) were infected with various doses of Ad5CMV.NINJ1 (VECTOR BIOLABS, Parkway, PA, USA). Forty-eight hours post infection, cells were transferred to a membrane for invasion and migration assays. Cells on the opposite side of the membrane were stained with hematoxylin and counted under a microscope.
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