Human Cancer Biology

HIC1 Modulates Prostate Cancer Progression by Epigenetic Modification

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Abstract

\textbf{Purpose:} Prostate cancer is the second leading cause of cancer deaths among men in Western counties, which has also occurred in Chinese males with markedly increasing incidence in recent years. Although the mechanism underlying its progression still remains unclear, epigenetic modifications are important ethological parameters. The purpose of this study is to determine the methylation status and function of hypermethylated in cancer 1 (HIC1) in prostate cancer progression.

\textbf{Experimental Design:} The methylation status of HIC1 promoter was assayed in cell lines, tissues, and plasma of patients with prostate cancer by using methylation-specific PCR and bisulfate sequencing PCR. The ability of HIC1 to regulate proliferation, migration, and invasion was assessed by MTT, scratch-healing assay, and reconstituted extracellular matrices in porous culture chambers. Tumorigenesis, metastases, and bone destruction were analyzed in mice bearing prostate cancer cells restoring HIC1 by using Xenogen IVIS with radiographic system and small-animal positron emission tomography computed tomographic images. Microarrays were searched for genes that had correlated expression with HIC1 mRNA. Reporter gene assays were used to determine whether HIC1 affected the expression of CXCR7, and chromatin immunoprecipitation was used to determine whether HIC1 bound to CXCR7 promoters. All \( P \) values were determined using 2-sided tests.

\textbf{Results:} The methylation status of 11 CpG sites within HIC1 promoter was abundantly methylated in cell lines, tissues, and plasma of patients with prostate cancer compared with those of respective normal controls. Restoring HIC1 expression in prostate cancer cells markedly inhibited proliferation, migration, and invasion and induced the apoptosis in these cells. Moreover, mice bearing prostate cancer–restoring HIC1 cells had a marked effect on reducing tumor growth, multiple tissue metastases, and bone destruction. Notably, we also identified that the chemokine receptor CXCR7 is a direct downstream target gene of HIC1. Finally, we showed that CXCR7 promoter in prostate cancer cells is negatively regulated by HIC1, which may be responsible for prostate cancer progression.

\textbf{Conclusions:} Our data show for the first time that hypermethylation of HIC1 promoter results in loss of its repressive function, responsible for prostate cancer progression and invasion. These findings suggest that therapies targeting epigenetic events regulating HIC1 expression may provide a more effective strategy for prostate cancer treatment.

\textbf{Introduction}

Prostate cancer is a common neoplasm. There were about 240,890 American men diagnosed with this disease and 33,720 deaths in 2011 (1). In China, although accurate epidemiologic data of the whole country were not available, prostate cancer incidence rates in developed areas (such as Shanghai, Beijing, Guangzhou, etc.) have been increasing dramatically during recent 10 years due, in part, to the wide use of screening test and adaptation of a Western style diet (2). Identifying new biomarkers responsible for prostate cancer progression may prove beneficial to prevent progression and metastasis and to provide an effective therapeutic strategy for this disease.

Hypermethylated in cancer 1 (HIC1) was originally isolated as a new candidate tumor suppressor gene because...
HIC1 Modulates Prostate Cancer Progression

Translational Relevance

This study aimed to further our understanding of the role that hypermethylated in cancer 1 (HIC1) plays in prostate cancer progression. Our data showed that HIC1 promoter hypermethylation was presented in cell lines, tissues, and plasma of patients with prostate cancer. In vitro assays, restoring HIC1 expression in prostate cancer cells by lentivirus virus or 5-aza-2'-deoxycytidine (decitabine) treatment, markedly inhibited proliferation, migration, and invasion and induced the apoptosis in these cells. Moreover, in vitro assays showed that the mice bearing prostate cancer–restoring HIC1 cells had a marked effect on reducing tumor growth, multiple tissue metastases, and bone destruction. These findings suggest that therapies targeting epigenetic events regulating HIC1 expression may provide a more effective strategy for prostate cancer treatment.

HIC1 in prostate cancer are further investigated. We also identified that chemokine receptor CXCR7 is a potential downstream target gene of HIC1. These findings for the first time indicate that HIC1 by hypermethylation may play a critical role in facilitating prostate cancer progression.

Materials and Methods

Some reagents, antibodies, and plasmids are listed in Supplementary Methods.

Cell cultures

The LNCaP, its metastatic subline C4-2B, and PC3 were obtained from the American Type Culture Collection and cultured in RPMI-1640 supplemented with 10% FBS (Invitrogen Corp). The human prostate epithelial cell line (PrEC; PriCells Co., LTD) was cultured in keratinocyte medium supplemented with 5 ng/mL human recombinant EGF and 50 µg/mL bovine pituitary extract (Invitrogen Corp).

Tissues and plasma of prostate cancer patients

Prostate cancer tissues and the respective peripheral blood were collected from patients and healthy controls at Shanghai Ruijin Hospital, Shanghai Jiao Tong University, and Shanghai the Ninth People’s Hospital of Tongji University (Shanghai, China) after the subjects’ informed consent and with institutional review board approval of the hospitals. All patients obtained a confirmed diagnosis of prostate cancer after resection.

Construction of lentiviral vectors

For restoring expression of HIC1 in PC3 and C4-2B cell lines, human full-length HIC1 cDNA was inserted into lentivirus vector pHHR-SIN-CSIGW (gift from Jun Mi, Shanghai Jiaotong University, Shanghai, China) under the control of SFFV promoter for stable expression. The fragment of HIC1 was inserted between SpeI and Ndel restriction sites according to the protocol. The construction was verified by sequencing. Supernatants were collected 36 to 48 hours after transfection, filtered through a 0.4-µm filter, and used directly to infect tumor cells. Two rounds of infection 8 hours apart were usually sufficient to infect more than 90% of cells. Transduced cells with high GFP levels were then isolated by fluorescence-activated cell sorting (FACS). The restored expression HIC1 in prostate cancer lines were respectively noted as PC3HIC1 and C4-2BHIC1 cells and the respective controls were noted as C4-2B GFP and PC3 GFP cells.

Methylation analysis and 5-aza-2'-deoxycytidine treatment

Methprimer software was used to forecast CpG island of upstream 1,000 bp promoter of transcription start site (TSS) in HIC1 gene subtype 1 and obtained methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) primers in CpG island. DNA from cell lines (treated with without 5-aza-2'-deoxycytidine), prostate cancer tissues, and plasma (0.2 mL) were purified using DNAzol (Invitrogen Corp).
and QIAMP DNA Blood Mini Kit (QIAGEN GmbH), treated with sodium bisulfite (ZYMEN Research Corp), and then analyzed by MSP or BSP (Supplementary Methods).

Quantitative real-time PCR
Total RNA was extracted from cells using TRIzol (Invitrogen Corp) and was reversely transcribed using the Reverse Transcription cDNA Synthesis Kit (Fermentas). cDNA was then used as template in the quantitative real-time PCR (qRT-PCR; Supplementary Methods).

cDNA microarray analysis
Agilent-014850 Whole Human Genome Microarrays 4 × 44K G4112F were used in this study. This chip targets >20,000 genes with >40,000 probes derived from a broad survey of well-known sources such as RefSeq, Genopat, Ensembl, Unigene, and others. The resulting view of the human genome covers 41K unique genes and transcripts that have been verified and optimized by alignment to the human genome assembly and by Agilent's Empirical Validation process. Total RNA (>300 ng) was extracted from 4 independent cultures of PC3GFP, PC3HIC1 cells and C4-2BHIC1, C4-2BHIC1 cells (Supplementary Methods).

Western blots analyses
Prostate cancer cell lines PC3, C4-2B were treated with and without 10 mmol/L of 5-aza-2'-deoxycytidine (5-aza-dC; Sigma) in 6.0-cm diameter plates (80%–90% confluency) and washed 3 times by PBS. Cells were lysed for 10 minutes on ice in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) containing an anti-protease mixture (Roche), and protein concentration was measured by BCA assays (Thermo Scientific). The protein fractions were resuspended in loading buffer and denatured at 100°C for 10 minutes. An aliquot of 5 to 15 μg of proteins was separated on 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked in 5% fat-free milk TBST buffer (0.1% Tween-20) for 2 hours at room temperature. For detection of glyceraldehyde-3-phosphohate dehydrogenase (GAPDH), HIC1, the membranes were either blocked in 5% fat-free milk TBST buffer (0.1% Tween-20), and 0.2 μg/mL of rabbit anti-human HIC1 polyclonal antibody (Catalog No: H8539, Sigma) were used in conjunction with 0.4 μg/mL of anti-species–conjugated horseradish peroxidase (HRP; Upstate) and detected by chemiluminescence (Amersham Pharmacia Inc). Similarly, for detection of MMP2/3, mouse anti-human MMP2 monoclonal antibody (0.2 mg/mL; sc-10736, Santa Cruz) and rabbit anti-human MMP3 polyclonal antibody (0.2 mg/mL; sc-21732, Santa Cruz) were used respectively.

Subcutaneous, intracardiac, intratibial, and tail vein injections
Subcutaneous tumors were established as described previously. Intracardiac, intratibial, and tail vein injections were conducted to determine the role that HIC1 plays in metastasis. After 4 weeks, bioluminescence was used to follow the prostate cancer–derived tissue metastases as the primary outcome. The mice were injected intraperitoneally with luciferin (200 μL at 15 mg/mL in PBS) before Xenogen IVIS™ (Supplementary Methods; ref. 15).

Small-animal PET-CT scanning
For the same purpose as above, the above mice injected PC3HIC1Luc and PC3GFPLuc in 2 months underwent 18F-FDG small-animal positron emission tomography (PET) and micro-computed tomographic (CT) in Shanghai Ruijin Hospital. PET-CT imaging was conducted on an Inveon MM Platform (Siemens Preclinical Solutions) with a computer-controlled bed and 8.5 cm transaxial and 5.7 cm axial fields of view (Supplementary Methods).

CXCR7 promoter luciferase assays
CXCR7 promoter truncation constructs have been previously described (18). The CXCR7 promoter region −813/−188, −386/−164, −191/−164, and −26/−164 constructs and the CXCR7 promoter −863/−168 XI mutants region containing −191/−164 and −26/−164 XI mutants were cloned into the pGL3 basic reporter gene vector to generate some CXCR7 promoter truncation constructs (Supplementary Methods).

Chromatin immunoprecipitation (ChIP) assay
Chromatin immunoprecipitation (ChIP) was conducted using an EZ-ChIP kit (Millipore Upstate). The dissociated DNA from immunoprecipitated protein/DNA complex was used for PCR assay (Supplementary Methods).

Statistical analysis
qRT-PCR and cell proliferation were analyzed using the Student t test, 2-tailed, with Excel software. The Kaplan–Meier log-rank test was used for analysis of mouse tumor development and survival data with GraphPad Prism 5 software. Statistical significance was defined as P < 0.05.

Results
Methylation status of HIC1 in prostate cancer
Emerging evidence indicates that HIC1, as a suppressor gene, is silenced by hypermethylation in many types of prevalent human tumors. Surprisingly however, the promoter hypermethylation of HIC1 in prostate tumors has not yet been studied in detail. Using Methprimer software indicating 3 CpG islands of upstream 1,000bp promoter of TSS in HIC1 gene subtype 1, we obtained the suitable MSP and BSP primers of −495 to −624 bp in CpG island 2 containing 11 CpG sites (Supplementary Fig. S1A). To explore whether the HIC1 promoter is methylated in prostate cancer, we analyzed its methylation status in prostate cancer tissues and cell lines by MSP and BSP, which covered the above promoter regions. As shown in Fig. 1A, 2 core promoter regions were markedly methylated in C4-2B and PC3 prostate cancer cells compared with the normal PrECs by MSP analyses. C4-2B, PC3, and PrECs were treated with 5 μmol/L 5-aza-dC for 4 days. PCR and Western blot assays
compared with the respective healthy control cases were 80.3% compared with the respective healthy control cases were 80.3%.

Moreover, the levels of methylated HIC1 promoter in 36 primary Chinese prostate cancer tissues were 11.3% (Fig. 1D, Supplementary Fig. S1B). The results showed that the mean methylated level of cancer tissues was restored only in prostate cancer cells, but no effect in PrECs (Fig. 1B). Next, the methylation status of 11 CpG sites in the HIC1 promoter from 36 prostate cancer tissues and the normal prostate tissues. Each row of circles represents each cell line. Each circle represents a single CpG site. Open circle represents unmethylated cytosine; filled circle represents methylated cytosine. The mean methylated level of cancer tissues compared with the respective healthy control cases were 80.3% ± 8.7% versus 31.56% ± 11.3%.

Figure 1. Methylation status of HIC1 CpG islands and the effects of 5-aza-dC on HIC1 expression. A, the core promoter region of HIC1 gene was methylated in 2 prostate cancer cell lines PC3, C4-2B (marked as M; 1 and 2 were both repetition sample hole), but unmethylated in the PrEC cell line (marked as U; 1 and 2 were both repetition sample hole) by MSP analysis. B, expression of HIC1 at mRNA (top) and protein (bottom) level was restored in PC3, C4-2B (marked as M; 1 and 2 were both repetition sample hole) by 5-aza-dC treatment for 4 days. C, schematic summary of 11 CpG sites in the HIC1 promoter region from -495 to -624 bp. Methylation analysis was conducted in 10 clones from each cell line. Each row of circles represents a single clone, and each circle represents a single CpG site. Open circle represents unmethylated cytosine; filled circle represents methylated cytosine. The prostate cancer cell lines showed higher methylation levels than the PrEC cell line as the control. The methylation ratios of 10 clones from each cell lines are summarized in the lowest bar chart. D, methylation status of 11 CpG sites in the HIC1 promoter from 36 prostate cancer tissues and the normal prostate tissues. Each row of circles represents occurred methylation status from 5 clones of a sample, and each circle represents a single CpG site. Open circle represents unmethylated cytosine; filled circle represents methylated cytosine. The mean methylated level of cancer tissues compared with the respective healthy control cases were 80.3% ± 8.7% versus 31.56% ± 11.3%.

indicated that the expression of HIC1 mRNA and protein was restored only in prostate cancer cells, but no effect in PrECs (Fig. 1B). Next, the methylation status of 11 CpG sites within the CpG island 2 of HIC1 by sequencing PCR products after bisulfite treatment was further assayed (Supplementary Fig. S1B). The results showed that the mean methylated percentage among 11 CpG sites of HIC1 promoter was greatly higher in C4-2B and PC3 cells than in PrECs (Fig. 1C). Moreover, the levels of methylated HIC1 promoter in 36 primary Chinese prostate cancer tissues compared with the respective healthy control cases were 80.3% ± 8.7% versus 31.56% ± 11.3% (Fig. 1D, Supplementary Table S1). Similar results were observed in 10 plasma DNAs from these patients (Supplementary Fig. S1C). These findings show that HIC1 promoter region is hypermethylated in prostate cancer.

Functional analysis after restoring HIC1 expression in prostate cancer cells

Because of silencing of HIC1 expression in prostate cancer cells by hypermethylation, we therefore restored its expression in C4-2B and PC3 cells by using lentivirus vector. Figure 2A showed that restored HIC1 expression was confirmed by Western blot assays in both cells (noted as PC3HIC1, C4-2BHIC1), which resulted in a proliferation arrest starting around 3 day compared with the controls (Fig. 2B). One possible explanation of the inhibited prostate cancer proliferation observed after having been infected
with HIC1 is that the gene may induce apoptosis in these cells. The loss of cellular membrane integrity as a reflection of cells undergoing apoptosis was determined by staining the cells for Annexin V. Indeed, HIC1 expression induced the apoptotic fraction in both cells compared with the controls (27.4 vs. 11.8% and 15.4 vs. 8.18%; Fig. 2C). The total number of dead or dying cells (54.7% C4-2BHIC1 vs. 15.3% C4-2BGFP, 32.6% PC3HIC1 vs. 16.4% PC3GFP) was increased as well.

The ability of HIC1 to regulate migration and invasion was assessed by scratch-healing assay and using reconstituted extracellular matrices in porous culture chambers. The results showed that the empty GFP vector–transfected cells nearly closed the wound at 24 hours after scratch, whereas HIC1-transfected cells were almost unable to heal the wound. The mean wound distances of the HIC1-transfected cells and the control cells at 24 hours were significantly different (435.231 μm vs. 125.28 μm; P < 0.001; Fig. 2D). In line with this finding, PC3HIC1 and C4-2BHIC1 cells resulted in less invasive capacity than in the respective controls (Fig. 2D). As shown in Fig. 2A, the effects may be ascribed to inhibiting MMP2/3 expressions after HIC1 expression, consistent with other group findings that MMP2/3 was responsible for tumor invasion by participating in some signal transduction pathways (19, 20). However, HIC1 knockdown in PC3 cells by siRNAs (Supplementary Fig. S2A) was capable of greatly increasing ability of its migration and invasion as

Figure 2. Effects after restoring HIC1 expression in prostate cancer cells. A, restoring HIC1 expression in PC3 and C4-2B cells (termed as PC3HIC1 and C4-2BHIC1 cells) was confirmed by Western blotting. Whole-cell lysates were immunoblotted with antibody to HIC1. The blots were stripped and reprobed with GAPDH antibody to confirm equal protein loading. B, proliferation of PC3 and C4-2B cells in response to restoring HIC1 expression. After a 24-hour serum withdrawal, prostate cancer cells were digested and washed 3 times in PBS, and 1 x 10⁶ cells were plated in into 96-well flat-bottomed tissue culture plates in 0.1 mL in complete growth medium. Proliferation was evaluated by XTT assay over a 4-day period.* significant difference from controls (P < 0.05, ANOVA) for means ± SE of n = 5 samples per condition. C, apoptosis profile of PC3 and C4-2B cells in response to restoring HIC1 expression. The role of HIC1 in promoting apoptosis in prostate cancer cells was evaluated by culturing equal numbers of cells in complete medium for 24 hours. Annexin-V–fluorescein staining and propidium iodide were used to identify apoptotic and necrotic cells by FACS analysis. D, left, restoring expression of HIC1 severely impaired cell migration. Serum was withdrawn before analysis to avoid effect of cell proliferation. The migration status was assessed by measuring the movement of cells into a scraped area created by a 10-μL pipette tube, and the spread of wound closure was observed at indicated times after scratching the surface of a confluent layer of cells. Scale bar, 100 μm. This experiment was carried out 3 times. Right, HIC1 regulates prostate cancer cell invasion. Prostate cancer cells were placed in the top chamber of invasion plates containing a reconstituted extracellular matrix in serum-free medium. Invasion was determined at 48 hours by MTT staining, and the data were read on a multiwell scanning spectrophotometer (Thermo Scientific) at A580 and presented as % invasion binding ± SD for n = 5. *, significant difference from controls (P < 0.05, ANOVA).
compared with the scrambled control (Supplementary Fig. S2B and S2C).

**Tumorigenicity and metastasis by HIC1 restoring expression in mice**

To evaluate that HIC1 plays a role in tumor growth, we transplanted PC3HIC1, C4-2BHIC1, and respective control cells to severe-combined immunodeficient (SCID) mice by subcutaneous injection. As shown in Fig. 3A and B, tumors burden from PC3HIC1 was significantly reduced compared with the control. Similar results were observed in mice from C4-2BHIC1 cells, albeit the effect was not as robust as above.

To further explore whether HIC1 modulate tumor metastasis, we firstly tagged PC3HIC1 cells and its control with luciferase gene by retrovirus infection. These tagged cells were then transplanted to BALB/c nude mice by intracardiac injection. The results showed that PC3HIC1 cells significantly reduced tissue metastases compared with PC3GFP cells regardless of whether the animal was imaged from ventral surface (Fig. 3C) and the dorsal surface (Supplementary Fig. 3A) in 4 weeks or from ventral surface (Supplementary Fig. S3B) and the dorsal surface in 8 weeks (Supplementary Fig. S3C). Examination of individual sites of bone metastasis also showed that HIC1 expression significantly reduced the total luminescent signal (i.e., total tumor burden) by radiographic analysis (Fig. 3C; Supplementary Fig. S3A–S3C). Moreover, representative fused small-animal PET and micro-CT images and immunohistochemical
staining further showed that HIC1 expression markedly inhibited multiple metastatic sites to liver, intestine, rhizine of thigh, pleural cavity, etc., in 8 weeks compared with the controls (Fig. 3D and Supplementary Fig. S3F). Similar results were also observed in nude mice bearing PC3HIC1 cells by tail vein injection in 4 weeks (Supplementary Fig. S3D) and 8 weeks (Supplementary Fig. S3E).

Bone destruction is the main cause of morbidity of patients with prostate cancer, which prompted us to assay the effect of HIC1 expression in bone microenvironment. Intratibial injection was therefore conducted in SCID mice using PC3HIC cells and the control PC3GFP cells. After 40 days, a radiographic and histologic analyses of the resulting tumors displayed extensive bone destruction in the control groups. In comparison, animals receiving PC3HIC1 cells had smaller lesions with less osteolytic damage (Supplementary Fig. S4A). Quantitative histomorphometry by tartrate-resistant acid phosphatase (TRAP) staining confirmed that the number of osteoclasts/mm of bone surface was lower in tissues resulting from the PC3HIC1 versus PC3GFP cells (2.26 ± 0.4 vs. 3.93 ± 0.2/mm; Supplementary Fig. S4B and S4C).

In brief, these findings strongly suggest that HIC1 expression had a significant effect on reducing osseous destruction by prostate cancer cells.

Identification of downstream genes by HIC1

To explore potential downstream targets induced by HIC1, we analyzed the genome-wide transcriptome profile of HIC1-restoring PC3 and C4-2B cells and the respective control cells by Agilent Whole Human Genome Microarrays. The microarray data set has been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42336). According to fold change (X 2.0) screening between expression of HIC1 and its respective control prostate cancer cells, 310 upregulated genes and 267 downregulated genes were commonly observed in PC3 and C4-2B cells. As shown in Fig. 4A, we picked up 36 cancer-associated genes for cluster mapping on the MeV microarray analysis platform (www.tm4.org/mev.html). We also identified genes related to
molecular function and picked up the top 36 gene sets that overlapped with different function clusters for exhibition (Fig. 4B and Supplementary Table S2).

CXCR7 expression modulated by HIC1 in prostate cancer

Among the genes markedly regulated in 2 prostate cancer cells with HIC1 expression, we paid attention to CXCR7 because our previous (21), and recent studies (22) showed that CXCR7 expression is closely associated with prostate cancer, bladder cancer, and breast cancer progression. In addition, CXCR7 has been described as a direct HIC1 target gene in U2OS cells overexpressing HIC1, and the regulatory region of CXCR7 promoter contained several functional HIC1 consensus–binding sites (HiRE, HIC1-responsive element; ref. 18). In this regard, we set out to explore the mechanisms through which CXCR7 expression is modulated by HIC1 in prostate cancer cells. Firstly, using qRT-PCR and FACS analysis confirmed that HIC1 expression markedly inhibited CXCR7 level in PC3 and C4-2B cells (Fig. 5A and B), consistent with the above microarray assays. Similar result was also observed in histologic staining of subcutaneous tumors with HIC1 expression (Supplementary Fig. S5A). Moreover, expression of CXCR7 mRNA and protein level was downregulated in term of restoring expression of HIC1 mRNA and protein by 5-aza treatment only in prostate cancer cells (Figs. 5C and D and 1B), but not in PrECs. In contrast, knockdown of HIC1 expression by siRNA1 and siRNA3 enhanced CXCR7 level in PC3 cells and PrECs (Supplementary Fig. S5B). Finally, high-density tissue microarrays were stained with both an anti-human CXCR7 and HIC1. Representative images are shown in Fig. 6A, indicating that expression of CXCR7 in cancer tissues was higher than the normal and prostatic intraepithelial neoplasia (PIN) tissues as reported previously (21). In contrast, HIC1 expression was markedly elevated in the normal and PIN tissues compared with the cancer tissues. Quantitative analysis confirmed the similar observations (Fig. 6B).

CXCR7 is a direct HIC1 target gene

On the basis of the above assays, these findings implied that CXCR7 may be reversibly modulated by HIC1. To determine the underlying mechanism, we used a series of CXCR7-truncated promoter/reporter fusion plasmids containing progressive 5’ deletions, which allowed us to conduct luciferase promoter reporter assays. These constructs were then transfected alone or with the pcDNA3-FLAG-HIC1 expression vector into PC3, C4-2B cells, and promoter activities were therefore measured in the absence or presence of HIC1. The data showed that, compared with these constructs transfection alone, transient transfection of HIC1

Figure 5. CXCR7 expression regulated by HIC1 in prostate cancer cells. A, CXCR7 mRNA expression was confirmed by quantitative real time-PCR in PC3HIC1 and C4-2BHIC1 cells. B, CXCR7 protein expression was evaluated by FACS analysis in PC3HIC1 and C4-2BHIC1 cells using mouse anti-human CXCR7 antibody and mouse anti-human IgG served as isotype control. C, CXCR7 expressions was decreased in PCA cells treated with 5-aza-CdR. Using PCR, expression of CXCR7 mRNA was assayed by restoring expression of HIC1 mRNA in presence of 5-aza treatment at 8 hours. D, CXCR7 expression was decreased in prostate cancer cells treated with 5-aza-CdR. Using FACS analysis, expression of CXCR7 protein level was assayed by restoring expression of HIC1 protein in presence of 5-aza treatment at 8 hours.
in PC3 cells markedly inhibited the CXCR7 promoter activity in the −813/+168, −386/+164, and −191/+164 constructs, even in the smaller construct −26/+164. The similar inhibitory effect was also shown in C4-2B cells (Fig. 6C). These results suggest that the regulatory region primarily involved in the HIC1-mediated repression of CXCR7 is located in the −26/+164 upstream region of the promoter that contains 1 of the 2 phylogenetically conserved and adjacent HIREs, such as sites IX and XI, as assayed by Van Rechem and colleagues (18). To verify the possibility, we used the AXI constructs in XI HiRE site with a mutation (TGCC into CAT) to abolish the ability of HIC1 binding. In line with the previous findings (18), the −813/+168, −191/+164, and −26/+164 constructs both display a high basal activity repressed by HIC1 expression. Notably, the mutated AXI constructs that the most significantly decreased repression ration by HIC1 was observed in the longest promoter construct −813/+168, but not in −191/+164 and −26/+164 constructs (Fig. 6D). These findings imply that the CXCR7 promoter is negatively regulated by HIC1.

To determine that CXCR7 is indeed a direct target gene of HIC1, we conducted ChIP assays using 100-kD molecular weight of HIC1 antibody (Sigma) and then analyzed the pull-down DNA. The primers were designed to amplify the region mediating the repressive effects of HIC1 on the −26/+164 site of CXCR7 promoter and containing adjacent HIREs as shown in the above assays (Supplementary Fig. S6A). As assay procedure control, we used primers located in the GAPDH promoter (Supplementary Fig. S6B). As shown in Supplementary Fig. S6A, CXCR7 was markedly amplified from the HIC1-immunoprecipitated PC3 and C4-2B chromatin compared with PrEC chromatin but not from
Chromatin immunoprecipitated by the control rabbit IgG. Taken together, these results show that endogenous HIC1 proteins are highly recruited onto the CXCR7 promoter in vivo both in PC3 and in C4-2B cells.

Discussion

HIC1 is a central transcriptional regulator of a few key genes controlling cell growth as well as cell death in response to p53-dependent apoptotic DNA damage through binding to SIRT1 promoter (8). Emerging evidence suggests that by combining with PATCHED, HIC1 may play an inhibitory role in Hedgehog pathway for medulloblastoma and capable of regulating Wnt pathway involved in function of stem cell (23). In fact, HIC1 is frequently hypermethylated as a result of silence or low-level in a variety of solid tumors and leukemia (13, 24), therefore making it a new therapeutic target for DNA methyltransferase inhibitors such as 5-aza-2′-deoxycytidine (decitabine; ref. 25).

This study aimed to further our understanding of the role that HIC1 plays in prostate cancer progression. Our data showed that HIC1 promoter hypermethylation was presented in Chinese patients with prostate cancer, potentially responsible for its progression, which has not been previously reported to our knowledge. Indeed, our study showed that restoring HIC1 expression in prostate cancer cells markedly inhibited proliferation, migration, and invasion and induced the apoptosis in these cells. In vitro experiments indicated that restoring HIC1 expression had a marked effect on reducing tumor growth, multiple tissue metastases, and osseous destruction in bone microenvironment. Microarray analysis and experiments in vivo identified chemokine receptor CXCR7 target gene as immediately downstream of HIC1.

CXCR7, formerly known as RDC1, has been shown to be a second receptor, in addition to CXCR4, for the chemokine CXCL12 (SDF-1, stoma cell–derived factor 1; refs. 21, 26). In the vasculature, the expression of CXCR7 is elevated in endothelial cells associated with tumors, and overexpression of CXCR7 in NIH 3T3 cells strongly supports a role for the receptor in tumorigenesis (27). More recently, CXCR7 expression has been shown to be elevated in endothelial cells associated with tumors. Membrane-associated CXCR7 is expressed on many tumor cell lines, on activated endothelial cells, and on fetal liver cells (26). Miao and colleagues (28) and our group further confirmed a critical role for CXCR7 in tumor vascular formation, angiogenesis, and promotion of the growth of breast (29), prostate cancer (21), and bladder cancer (22) in vivo. Targeting CXCR7 by various strategies such as short hairpin RNA (shRNA) interferences, specific high-affinity small-molecule antagonist or intrakinase severely reduces proliferation of carcinoma cells in vitro (22, 26) as well as in vivo tumor growth in animal models (30). These characteristics suggest that CXCR7 plays a role in regulating immunity, angiogenesis, stem cell trafficking, and mediating organ-specific metastases of cancer (26–30). However, the mechanism underlying CXCR7 expression modulated by immediately upstream targets still remains unsettled.

In this study, we further showed that endogenous HIC1 could bind to the promoters of CXCR7, thus resulting in inhibiting its effect. Interestingly, the mutated ΔX1 constructs that the most significantly decreased repression ratio by HIC1 was only observed in the longest promoter construct −813/+168, but not in −264/+164 constructs. To be fully understood, the mechanism underlying the effect of HIC1 in CXCR7 expression is warrant investigation. However, these findings imply that the CXCR7 promoter is negatively regulated by HIC1. Indeed, CXCR7 expression in prostate cancer tissue is negatively modulated by HIC1, as a critical upstream of CXCR7, which may participate in prostate cancer progression.

Notable, our initial findings suggest that although HIC1 expression levels decreased by epigenetic modification during the prostate cancer development, which inhibited CXCR7 influence on prostate cancer progression. But hypermethylation did not consistently correlate significantly with HIC1 expression levels in breast cancer cells, non–small cell lung cancer cells compared with the corresponding non–cancerous tissue, indicating that other inhibitory mechanisms other than hypermethylation of the HIC1 promoter may exist, for example, SUMOylation or acetylation modification of the promoter, mutations/inactivation of the positive HIC1 regulator p53, or aberrant expression of not yet identified HIC1 inhibitors (31, 32). However, to be fully understood, the function of HIC1 in cancer biology is warrant investigated.

In summary, here we present that HIC1 by epigenetic modification may participate in prostate cancer progression, suggesting that therapies targeting epigenetic events regulating HIC1 expression may provide a more effective strategy for prostate cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Wang, J. Zheng, X. Sun, J. Zhang
Development of methodology: J. Zheng, X. Sun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zheng, X. Sun, T. Ding, M. Zhao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zheng, J. Wang, X. Sun, T. Ding, G. Xiao
Writing, review, and/or revision of the manuscript: J. Zheng, J. Wang, X. Sun, T. Ding, J. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Zheng, J. Wang, X. S, M. Hao, T. Ding, D. Xiong, X. Wang, Y. Zhu, C. Cheng
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