Stable Suppression of Tumorigenicity by Pin1-Targeted RNA Interference in Prostate Cancer

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Abstract

Purpose: The peptidyl-prolyl isomerase Pin1 plays a catalytic role in oncogenesis in solid cancers, including prostate cancer. In the present study, we sought to determine the potential of Pin1-targeted gene silencing in inhibiting cellular growth and tumorigenicity in prostate cancer. Experimental Design: A retrovirus-mediated RNA interference targeting Pin1 was expressed in PC3 and LNCaP cells, and cell growth and several transformed properties were investigated. Results: The stable expression of Pin1-specific small interfering RNA constructs in PC3 and LNCaP cells significantly reduced cellular proliferation, colony formation, migration, and invasion but strongly enhanced the apoptotic response induced by serum depletion or treatment with anticancer agents. Furthermore, Pin1 depletion significantly suppressed tumorigenic potential in athymic mice, resulting in the inhibition of both tumor growth and angiogenesis. Conclusions: These results strongly suggest that Pin1 plays an important role not only in tumorigenesis but also in the maintenance of the transformed phenotype in prostate cancer cells. Hence, Pin1 may serve as a promising therapeutic target, particularly for recurrent prostate tumors.

Prostate cancer is one of the most common male tumors and its incidence is increasing steadily worldwide (1). Despite the availability of an earlier diagnosis using serum prostate-specific antigen and improved treatments with hormone therapy and surgery, prostate cancer is still the leading cause of male cancer death in many countries (2, 3). Radical prostatectomy is a definitive form of therapy for clinically localized prostate cancers but a substantial number of cases have shown tumor recurrence even when tumors have been localized pathologically to the prostate (3). These recurrent cancers are also generally hormone independent and are refractory to treatments with chemical reagents and radiation (4, 5). Although our understanding of the molecular biology of prostate cancer has increased in recent years, a detailed knowledge of the molecular mechanisms underlying its formation and progression remains elusive. Furthermore, no specific molecular targets for use in clinical practice have yet been identified (6). It will be desirable in the future to develop such potent molecular targets for novel clinical treatments based on the etiology of prostate cancer formation.

The peptidyl-prolyl isomerase Pin1 is an enzyme that specifically binds phosphorylated serine or threonine, immediately preceding proline (pSer/Thr-Pro), in a subset of proteins and then regulates their function by promoting cis/trans isomerization of the peptide bond (7–9). Pin1 contains both an NH2-terminal WW domain and a COOH-terminal isomerase domain (9). The WW domain binds specific pSer/Thr-Pro motifs to enable the isomerase domain to function by inducing conformational changes to the pSer/Thr-Pro bond. Such conformational changes have been shown to have profound effects on the function of Pin1 substrates by modulating catalytic activity, phosphorylation status, protein-protein interactions, subcellular localization, and protein stability (9). Consequently, Pin1 has been shown to be involved in the regulation of many cellular events, including proliferation and differentiation, and has been reported to be highly overexpressed in a number of human malignancies, including breast and prostate cancers (10–13). Significantly, Pin1 enhances several oncogenic signaling pathways and facilitates both cellular proliferation and oncogenesis. Pin1 has now been shown to activate the Ras/c-Jun, retinoblastoma/E2F, wnt/β-catenin, and nuclear factor κB pathways, all of which play central roles in tumorigenesis and malignancy (11, 14–17). These findings indicate that Pin1-mediated postphosphorylation regulation plays a pivotal role in both cellular proliferation and transformation.

Although the detailed molecular mechanism(s) remains to be elucidated, several studies have now implicated Pin1 in the genesis and malignancy of prostate cancer (10, 12). Ayala et al. (12) did a comprehensive immunohistochemical analysis and showed that Pin1 is highly overexpressed in a number of prostate cancer cases and that Pin1 expression levels were
positively correlated with some clinicopathologic variables in patients and could be an independent prognostic factor. These observations indicated that Pin1 may serve as a mediator of malignant behavior in prostate cancer and suggested that the inhibitory targeting of Pin1 might be incorporated into novel prostate cancer therapies. However, it is not known whether Pin1 depletion would affect actual cellular growth or would block tumorigenic properties in prostate cancer.

Here, we show that Pin1 plays an important role not only in cellular growth but also in the maintenance of the tumorigenic properties of prostate cancer. Retrovirus-mediated small interfering RNA (siRNA) targeting of Pin1 resulted in the stable suppression of both cell growth and tumorigenic phenotypes including cell migration, invasion, and angiogenesis in prostate cancer cells. These results indicate that Pin1 plays a crucial role in a range of tumorigenic properties in prostate cancer cells and could be adopted as a molecular target for the future development of treatments for prostate cancer and other malignancies.

Materials and Methods

**Retrovirus-mediated small interfering RNA constructs.** pSUPER-internal ribosome entry site-puro vector was digested with BglII and HindIII and annealed oligos (Pin1: 5'-gatccGCGGCTGTACTCTTCAAtgattgtggtgagga-3'; control: 5'-gatccTCGTATGTTGTGTGAATTttcaagagaAATTCCACACAATACGAttttttggaaa-3') were ligated into this vector (18). For the production of retroviruses, PLAT-E cells were transfected with pSUPER and vesicular stomatitis virus G vectors using FuGENE 6 transfection reagent (Roche Applied Science, La Jolla, CA) as previously described (19). Culture supernatants of PLAT-E cells were collected 60 hours after transfection with retroviral vectors. Target cell lines were infected in the presence of 10 µg/mL Polybrene.

**Construction of small interfering RNA–resistant Pin1 expression vector.** siRNA-resistant wild-type Pin1 (pCDNA-Pin1*) or its pep-tidyl-prolyl isomerase mutant (pCDNA-K63A*) was generated with site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the instruction of the manufacturer. The forward primer sequence was 5'-gcagctcgcgcgggttttatttttcaccaacatcaac-3'.

**Cell growth, retrovirus infection, and gelatin zymography.** Human prostate cancer cell lines PC3 and LNCaP were cultured in DMEM or RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin. Cells were treated with retrovirus-mediated pSUPER-puro encoding nonspecific or Pin1-specific siRNA and selected by continuous growth in puromycin (0.5 µg/mL) to isolate stable clones (19). For zymography for matrix metalloproteinase (MMP) activity, cell culture medium was concentrated 50-fold and loaded on a 7.5% zymogram gel as previously described (20).

**Immunoblot analysis.** Twenty micrograms of protein lysates were separated by 7.5% or 15% SDS-PAGE and immunoblotted with anti-Pin1 polyclonal (11), anti-α-tubulin monoclonal (Sigma, St. Louis, MO), anti–vascular endothelial growth factor (VEGF)-A, and anti–VEGF-C polyclonal (Santa Cruz, Santa Cruz, CA) antibodies as previously described (15).

**Senescence-associated β-galactosidase assay.** Cultured cells were fixed in 3% formaldehyde/0.2% glutaraldehyde in PBS (pH 7.4) at room temperature for 10 minutes. Cells were washed and incubated at 37°C with 5-bromo-4-chloro-3-indolyl β-galactoside (X-gal) in citric acid/sodium phosphate buffer (pH 5.7) for 12 hours according to the method of Dimri et al. (21).

**Transwell migration assay.** Cells were plated at a density of 2 × 10^5 cells in 24-well culture plates and migration assays were done using a chemotaxis chamber (BD Biosciences, San Jose, CA) and transwell tissue culture plates (8 µm pore size). The bottom of the chamber was coated with either 10 µg/mL fibronectin or Matrigel (BD Biosciences). One-hundred microliters of a 10^5 cells/mL suspension were introduced into each well and allowed to migrate for 6 hours. Cells were then fixed with methanol and stained with crystal violet.

**Wound healing assay.** Cells were seeded at a density of 10^6 in six-well plates and cultured for 24 hours. A wound was made using the tip of a pipette as previously described (20). After 8 hours, the cells were fixed with 3% formaldehyde in PBS for 15 minutes and images of the wound were generated using different fields.

**Soft agar colony formation assay.** Soft agar assays were done by seeding cells at a density of 10^4 in 60-mm tissue culture dishes containing 0.3% top low-melt agarose-0.5% bottom low-melt agarose as previously described (14). Cells were fed every 4 days and colonies were counted and measured after 2 weeks.

**Xenograft studies.** PC3 and LNCaP cells were washed twice with antibiotic-free and serum-free DMEM and finally resuspended at a density of 5 × 10^6 cells in 0.2 mL. The cell suspension was mixed with equal volume of Matrigel (BD Bioscience) and injected s.c. into 6-week-old nude athymic BALB/c mice, which were sacrificed after 6 to 8 weeks. Tumors were excised and weighed, fixed for 24 hours in 10% formalin, and washed with PBS. The tumor specimens were then subjected to paraffin section followed by the immunohistochemistry with anti–factor VIII (Nichirei, Tokyo, Japan) or anti–VEGF-C antibody (R&D Systems, Minneapolis, MN) as previously described (15, 22).

Results

**Stable expression of Pin1-specific small interfering RNA affects cell growth and morphology.** Ayala et al. (12) have shown that Pin1 expression levels were tightly correlated with both a higher probability and a shorter period of tumor recurrence following radical prostatectomy by a comprehensive immunohistochemical analysis. Furthermore, our unpublished observation indicated that Pin1 mRNA levels are also up-regulated in a population of prostate cancer, as correlated with Pin1 protein levels, and positively associated with a higher rate of tumor recurrence after the surgery. These results of clinical samples prompted us to examine whether targeted Pin1 inhibition would affect the proliferative and malignant properties of prostate cancer cells. For this purpose, we constructed retrovirus-mediated siRNA vectors that targeted Pin1 mRNA to enable stable suppression of Pin1 expression. We used two representative prostate cancer cell lines, PC3 and LNCaP, in which Pin1 is highly overexpressed than in normal human prostate epithelial cells (Fig. 1A). We selected infectants with puromycin for 72 hours and then determined the Pin1 protein levels by immunoblot analysis. As shown in Fig. 1B, Pin1 protein levels were significantly reduced in cells expressing Pin1 siRNA (>95% reduction in endogenous levels) but not in cells expressing control siRNA when compared with parental cells.

We next evaluated the effects of Pin1 depletion on in vitro cell growth and morphology. The growth rate was measured in six-well plates for up to 4 days. Suppression of Pin1 by siRNA resulted in a substantial decrease in growth rate but cells expressing control siRNA vectors grew normally in these prostate cancer cells (Fig. 1C). We next examined the effect of Pin1 siRNA on normal fibroblast cells. Both WI-38 and TIG-1 fibroblast cells were infected with either control of Pin1-specific siRNA-retrovirus vector and were selected with puromycin followed by cell growth analysis. As shown in Fig. 1D, Pin1 inhibition did not affect cell growth in both WI-38 and TIG-1 fibroblast cells, suggesting the specific effect of Pin1 suppression on cancer-specific cell growth.
Morphologic analysis also revealed that cells expressing Pin1 siRNA became relatively larger with vacuolated nuclei and a granular cytoplasm, thus showing specific features of senescent cells. Senescence-associated β-galactosidase staining of these cells further revealed significant increases in the number of senescence-associated β-galactosidase–positive cells in the Pin1 siRNA population but not in control siRNA cells (Fig. 2A; ref. 21). A BrdUrd incorporation experiment also indicated a significant decrease in the S-phase population in Pin1 siRNA cells but not in control siRNA cells (Fig. 2B). Furthermore, forced expression of a wild-type Pin1, but not its peptidyl-prolyl isomerase mutant (K63A), which was not subject to knockdown, reverted the growth of cells carrying Pin1 siRNA cells up to the level of the control siRNA cells (Fig. 2B). These results indicate that the specific depletion of Pin1 affects both cell proliferation and morphology by partly inducing cellular senescence in prostate cancer cells.

Loss of Pin1 expression abrogates anchorage-dependent and -independent colony formation in prostate cancer cells. To examine the effects of Pin1 suppression on tumorigenic phenotypes in prostate cancer cells, we examined the effects of Pin1 suppression on either anchorage-dependent or -independent colony formation. PC3 cells infected with either control or Pin1-specific siRNA constructs were seeded at a density of $10^3$ cells in 100-mm diameter dishes. Cells expressing Pin1 siRNA produced 3-fold fewer colonies than parental or control cells (Fig. 2C). Similar results were also observed in LNCaP cells except that the colony size was much smaller in this case (data not shown).

We then evaluated the effect of Pin1 suppression on anchorage-independent colony formation in soft agar as an additional assessment of tumorigenicity in vitro. Pin1 siRNA expression significantly abrogated anchorage-independent growth in which both colony number and size are much reduced (Fig. 2D). However, cells expressing control siRNA vector did...
not show any such loss in colony formation ability when compared with parental cells. Taken together, these results indicate that inhibition of Pin1 by a specific siRNA treatment suppresses the colony-forming ability of prostate cancer cells in vitro.

**Pin1 suppression effects on migration and wound healing in prostate cancer cells.** We next investigated whether stable siRNA expression modifies extracellular matrix interactions and causes an increase or a decrease in cell migration. It has been shown that prostate cancers with high Pin1 expression often metastasize to lymph nodes or other organs (12). We tested cellular migration and invasion levels using transwell chambers coated with either fibronectin or Matrigel as previously described (20). As shown in Fig. 3A, cells stably expressing Pin1 siRNA show a significant decrease in migration on both substrates whereas no effect was seen in control siRNA–expressing cells.

Fig. 2. Pin1 depletion induces cellular senescence and suppresses both anchorage–dependent and–independent colony formation. A, the indicated PC3 cells were treated with senescence–associated β–galactosidase staining. The average number of senescence–associated β–galactosidase–positive cells is depicted at the right lower corner. B, percentage of S-phase cell populations determined by BrdUrd incorporation. PC3 cells carrying Pin1 siRNA were further transfected with either vector control, siRNA–resistant wild-type Pin1 (Pin1*), or its peptidyl–prolyl isomerase mutant (K63A*). Top, Pin1 expression analyzed by anti–Pin1 immunoblotting. The indicated PC3 cells were treated with BrdUrd for 3 hours, immunostained with anti–BrdUrd monoclonal antibody, and analyzed by fluorescence microscopy. Bottom, the numbers of BrdUrd–positive cells were scored from >300 cells in three independent experiments (columns, mean; bars, SD). *, Pin1 constructs of which codons were optimized to remove siRNA elements. C, equal numbers of PC3 cells (10^5) expressing either control siRNA or Pin1 siRNA were seeded into 10–cm plastic dishes. After 14 days, the cells were fixed and stained with crystal violet. The numbers below the plates indicate colony numbers (mean ± SD) from three independent experiments. D, cells were plated in 0.3% soft agar and cultured for 2 weeks. After 14 days, colony formation was scored microscopically. Colony numbers (columns, mean; bars, SD) were calculated from three independent experiments.
involved in invasion and metastatic process of prostate cancer (26, 27), these results indicate that targeted Pin1 inhibition by siRNA may suppress cell migration and invasion partly via down-regulation of MMP-2 expression in prostate cancer cells.

**Pin1 inhibition sensitizes cells to apoptosis induced by serum depletion and by treatment with anticancer drugs.** One of the current major issues in prostate cancer treatment is therapeutics against hormone refractory prostate cancer. We therefore wished to address the question of whether Pin1 inhibition affects the apoptotic response in prostate cancer cells following either serum depletion or treatment with anticancer drugs. First, we cultured the siRNA-transfected PC3 cells in serum-depleted medium for 60 hours. Although the expression of Pin1 siRNA did not affect cellular viability in normal medium, these cells underwent apoptosis in serum-depleted medium (Fig. 4A). However, parental PC3 cells and control siRNA–expressing PC3 cells were not affected in this way by serum depletion (Fig. 4A). These results indicate that Pin1-inhibited prostate cancer cells are likely to undergo apoptosis with insufficiency in growth factors or nutrients.

We next examined whether Pin1 inhibition affects the sensitivity to chemotherapeutic agents in prostate cancer cells. A major anticancer agent used in the treatment of prostate cancer is the microtubule-disorganizing agent Taxol. We treated our siRNA-transfected cells with different concentrations of Taxol for 36 hours and, as shown in Fig. 4B and C, Pin1 suppression significantly sensitized cells to Taxol-induced cell death as revealed by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay and staining with Hoechst 33342 dye. Interestingly, these cells became much larger in size and developed giant nuclei with focal chromosomal condensation, indicating possible mitotic catastrophe (Fig. 4C). Similar results were also observed in cells treated with another microtubule disorganizing agent, Nocodazole (data not shown). These data indicate that targeted Pin1 inhibition sensitizes prostate cancer cells to apoptosis induced by serum depletion and microtubule-disorganizing agents.

**Pin1-specific small interfering RNA abrogates cancer cell growth in vivo.** To determine whether Pin1 suppression decreases in vivo tumorigenicity, xenograft studies were performed.
conducted using PC3 and LNCaP cells stably expressing either control or Pin1-specific siRNA. Six mice were injected with $5 \times 10^6$ cells mixed with 50% Matrigel. Both prostate cancer cell transfectants expressing Pin1-specific siRNA showed a decrease in tumor size and weight when injected into nude athymic mice, compared with control cells (Fig. 5A-D). In addition, the mean tumor size in Pin1 inhibited cells was maintained at significantly smaller levels than tumors in control siRNA mice (Fig. 5C). These data provide further evidence that Pin1 suppression abrogates oncogenic potential in vivo.

The growth of tumors beyond a minimal size has been hypothesized to be dependent on the induction of new blood vessel growth, or angiogenesis, which in turn supplies needed nutrients to rapidly dividing tumor cells (28). We thus analyzed the effects of Pin1 inhibition on blood vessel growth/angiogenesis in mice. Six weeks after injection, tumors were harvested from mice injected with control and Pin1 siRNA PC3 cells (Fig. 5C). Paraffin-embedded sections of five different tumors were processed and stained for factor VIII, an endothelial specific marker (29). As shown in Fig. 6A, these sections showed a dramatic increase in microvessel formation throughout the cell cords in control siRNA-expressing tumor tissues whereas the tumors expressing Pin1 siRNA had much less microvessel formation.

Several studies have previously indicated that VEGFs are critical factors in the development of new blood vessels and angiogenesis (30). This prompted us to evaluate the effect of Pin1 suppression on VEGF expression. Both immunohistochemistry and immunoblot analyses subsequently revealed decreases in VEGF-A and VEGF-C protein levels in xenograft cancer tissues composed of Pin1 siRNA cells but not in those containing control siRNA-expressing cells (Fig. 6B). Because VEGFs are responsible for the proliferation and induction of endothelial cells that line the blood vessels and lymphatic ducts, these results indicate that inhibition of Pin1 is likely to suppress tumor growth partly by suppressing microvessel formation and angiogenesis via down-regulation of VEGFs.

**Discussion**

We here report that specific Pin1 depletion by retrovirus-mediated siRNA results in decreased tumor growth and suppresses a range of tumorigenic phenotypes in prostate cancer cells. Hanahan and Weinberg (31) proposed that the vast catalogue of cancer cell phenotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth. These include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes can be acquired during and after tumor development. In line with this idea, we attempted to design experiments to verify whether Pin1 inhibition affects these cancer-specific phenotypes in prostate cancer cells. We showed in the current study that (a) Pin1 depletion suppresses tumor cell growth, accompanied by the partial induction of cellular senescence; (b) colony formation abilities on plastic plates or in soft agar are significantly suppressed by Pin1 inhibition; (c) cell migration and invasion are inhibited in Pin1-depleted cells,
which was then confirmed by transwell and wound healing assays; (d) cellular sensitivity to apoptosis, induced by either serum depletion or microtubule-disorganizing agents, is increased also by the suppression of Pin1; and (e) tumor growth was suppressed by the loss of Pin1 in athymic nude mice with decreased blood vessel formation observed in tumors. These results first showed that Pin1 depletion affects not only tumor cell growth but also a broad range of tumorigenic phenotypes in prostate cancer cells both *in vitro* and *in vivo*.

One of the current major issues in prostate cancer treatment is resistance to chemotherapy. We have shown that Pin1 inhibition sensitizes prostate cancer cells to apoptosis induced by microtubule disorganizing agents such as Taxol. In this case, Pin1-depleted cells show escape from the spindle checkpoint and develop into multinucleated giant cells with characteristic aneuploidy that results in apoptosis, which is a main feature of mitotic catastrophe (32). Induction of apoptosis in cancer cells via Pin1 inhibition would be an appealing therapeutic strategy because many recurrent prostate cancers are refractory to chemotherapeutic agents or radiation therapy (4). Because Pin1 inhibition sensitizes cells to drugs that induce the spindle checkpoint, a combination therapy using a Pin1 antagonist in concert with microtubule organizing agents could be an effective treatment strategy for recurrent prostate tumors.

VEGF is a multifunctional growth factor that plays a major role in the initiation of both angiogenesis and lymphangiogenesis by acting directly as a mitogenic and chemotactic factor for endothelial cells (30, 33, 34). In this study, we first show that Pin1 depletion inhibited tumor angiogenesis accompanied by the down-regulation of VEGF-A and VEGF-C in the xenograft study of athymic nude mice. Expression of VEGF-C in human prostate cancer is associated with regional lymph node metastasis by inducing lymphangiogenesis (35, 36). In fact, Ayala et al. showed that prostate cancers with high Pin1 expressions often exhibit regional lymph node metastasis (12). Thus, specific Pin1 inhibition may be an appealing strategy to suppress microvessel formation and lymphangiogenesis in cancer tissue.

It is well established that up-regulation of MMP activities results in the proteolytic degradation of the extracellular matrix and the basement membrane, which promotes tumor growth and metastasis (24). Our interesting observation was that inhibition of Pin1 causes the suppressed expression of MMP-2 in prostate cancer cells. MMP-2 is specifically activated in a large population of prostate cancer cells and is involved in tumor invasion and metastasis (26, 27). Although further detailed analyses would be necessary, our results indicate that Pin1 suppressed tumor metastasis at least in part by suppressing MMP-2 expression.

From a clinical point of view, cancer cells possess multiple characteristics that distinguish them from normal cells (31). These characteristic differences, however, may be used as targets for novel anticancer therapies (13, 17). In addition, many cancer cells seem to be dependent on Pin1 for maintenance of cell growth and a tumorigenic phenotype.

**Fig. 5.** Pin1 siRNA expression decreases tumorigenicity in nude mice. A, athymic nude mice received injections of 5 × 10⁶ PC3 or LNCaP cells mixed with an equal volume of Matrigel. The cell suspension was injected s.c. into nude mice. Representative tumor formations at 6 weeks after injection. B, mean tumor sizes for four animals up to 6 weeks after injection. C, representative appearance of tumor mass resected from athymic nude mice at 6 weeks after injection. D, columns, mean tumor weight at 6 weeks after injection; bars, SD.
through multiple phosphorylation-dependent intracellular signaling pathways (13). Importantly, Pin1 depletion did not affect normal cell growth and cellular function in noncancerous fibroblasts as shown in this study. Furthermore, Pin1 knockout mice develop normally and do not show any significant phenotype at a young age although they exhibit a phenotype related to cell proliferation insufficiency in testis, retia, and mammary glands (22, 37). However, prostate tissues are not affected in Pin1 knockout mouse (22)

A.Ryo and K.P. Lu, unpublished observations.

Fig. 6. Pin1 depletion suppresses tumor angiogenesis in nude mice. A, tumor tissues (PC3-origin) from athymic nude mice were stained with either anti—factor VIII or anti—VEGF-C polyclonal antibodies followed by 3,3-diaminobenzidine staining (brown) and hematoxylin staining (blue). Note that immunohistochemical analysis with anti—factor VIII polyclonal antibody shows rarefaction of tumor blood vessels in Pin1 siRNA xenografts. B, down-regulation of VEGFs in Pin1-depleted xenografts in nude mouse. Tissue lysates were subjected to immunoblot analysis with anti—VEGF-A, anti—VEGF-C, and anti-tubulin antibodies.

In summary, we have shown in this report that targeted Pin1 inhibition by specific siRNA prevents cell proliferation and suppresses transformed properties in prostate cancer cells. Further studies using in vivo mouse models of prostate cancer with specific Pin1 inhibitors should provide further insights into the significance of Pin1 inhibition for the future treatment of prostate cancer.

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