Human heterochromatin protein 1 isoforms regulate androgen receptor signaling in prostate cancer

Momoe Itsumi1, Masaki Shiota1, Akira Yokomizo1, Eiji Kashiwagi1, Ario Takeuchi1, Katsunori Tatsugami1, Junichi Inokuchi1, YooHyun Song1, Takeshi Uchiumi2 and Seiji Naito1

1Departments of Urology and 2Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Correspondence should be addressed to A Yokomizo
Email yokoa@uro.med.kyushu-u.ac.jp

Abstract

Androgen receptor (AR) signaling is critical for the tumorigenesis and development of prostate cancer, as well as the progression to castration-resistant prostate cancer. We previously showed that the heterochromatin protein 1 (HP1) β isoform plays a critical role in transactivation of AR signaling as an AR coactivator that promotes prostate cancer cell proliferation. However, the roles of other HP1 isoforms, HP1α and HP1γ, in AR expression and prostate cancer remain unclear. Here, we found that knockdown of HP1γ, but not HP1α, reduced AR expression and cell proliferation by inducing cell cycle arrest at G1 phase in LNCaP cells. Conversely, overexpression of full-length HP1α and its C-terminal deletion mutant increased AR expression and cell growth, whereas overexpression of HP1γ had no effect. Similarly, HP1α overexpression promoted 22Rv1 cell growth, whereas HP1γ knockdown reduced the proliferation of CxR cells, a castration-resistant LNCaP derivative. Taken together, HP1 isoforms distinctly augment AR signaling and cell growth in prostate cancer. Therefore, silencing of HP1β and HP1γ may be a promising therapeutic strategy for treatment of prostate cancer.

Key Words
- androgen receptor
- castration-resistant prostate cancer
- heterochromatin protein 1
- prostate cancer

Introduction

Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer-related death of men in developed countries (Siegel et al. 2012). Prostate-specific antigen (PSA) screening has improved early detection of prostate cancer and has recently been proved to reduce the risk of death by prostate cancer (Schro¨der et al. 2009, 2012, Hugosson et al. 2010, Andriole et al. 2012). However, a quarter of early-stage prostate cancer patients still suffer from relapse of the disease despite surgical and/or radiation therapy. In addition, many patients with prostate cancer are diagnosed at an advanced stage of the disease. Although most prostate cancers are originally androgen dependent and respond well to androgen-deprivation therapy (ADT), they eventually become castration-resistant prostate cancer (CRPC) during ADT, which is thought to remain dependent on androgen receptor (AR) signaling for growth in a low-androgen milieu (Ryan & Tindall 2011).

The AR is a member of the class I subgroup of the nuclear receptor superfamily and a ligand-dependent transcription factor. The androgen/AR signaling pathway is thought to play a central role in the development and
progression of prostate cancer including CRPC. The mammalian heterochromatin protein 1 (HP1) family plays a critical role in a variety of cellular processes including centromere stability, telomere stability, regulation of gene expression, DNA repair, cellular senescence, and cancer progression through their activities in DNA and its interacting proteins (Zhang & Adams 2007, Dialynas et al. 2008, Ayoub et al. 2009). Three mammalian HP1 isoforms, HP1α (CBX5), HP1β (CBX1), and HP1γ (CBX3), have been identified in humans. Each of these heterochromatinins contains two conserved domains, a chromodomain (CD) interacting with dimethylated Lys9 of histone H3 and a chromoshadow domain (CSD) interacting with the PxVXL motif of its partner, which are separated by a flexible hinge region (Kwon & Workman 2011). HP1 isoforms are known to exhibit different subnuclear localizations in interphasic nuclei. HP1α is mainly centromeric, HP1β is also centromeric, but to a lesser extent, and HP1γ is located in both euchromatic and heterochromatic compartments (Dialynas et al. 2007). HP1 heteromers are associated with nucleosomal core histones (Zhao et al. 2000) and reduce the transcription of nearby promoters when tethered to DNA (Cryderman et al. 1999). Furthermore, HP1 isoforms in mice and humans interact directly with the transcriptional corepressor TIFβ, supporting the hypothesis that HP1 isoforms play a role in gene silencing (Nielsen et al. 1999). However, it has been recently reported that HP1 isoforms may work at euchromatic regions and as a gene activator despite their name, localization, and suggested function (Piacentini et al. 2003, Hediger & Gasser 2006, de Wit et al. 2007, Fanti & Pimpinelli 2008). On the other hand, in several cancers, HP1α expression has been reported to be decreased compared with that in the corresponding normal tissues (Pomeroy et al. 2002, Wasenius et al. 2003). In addition, reduction of HP1α expression has been observed in various cancers including cancers undergoing progression (Dialynas et al. 2008). However, several recent reports show that HP1 isoforms are upregulated in various tumor tissues, compared with

**Figure 1**

HP1γ knockdown transcriptionally reduces AR expression. (A) LNCaP cells were transfected with 40 nM of the indicated siRNA. At 72 h post-transfection, quantitative real-time PCR was performed using the indicated primers and probes. The target transcript level was normalized to the GAPDH transcript level. All values represent at least three independent experiments. The level of each transcript from cells transfected with control siRNA was defined as 1. Boxes, mean; bars, ± S.D. *P<0.05 (compared with control siRNA). (B) LNCaP cells were transfected with 40 nM of the indicated siRNA. At 72 h post-transfection, whole cell extracts were subjected to western blotting using the indicated antibodies. (C) LNCaP cells were transfected with 0.5 μg/ml of the AR-Luc #3 reporter plasmids, 20 nM of the indicated siRNA, and 0.05 μg/ml pRL-TK and then incubated for 48 h. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. All values represent at least three independent experiments. The luciferase activity in cells transfected with control siRNA was defined as 1. Boxes, mean; bars, ± S.D. *P<0.05 (compared with control siRNA).
those in normal tissues, and increases according to tumor progression (De Koning et al. 2009, Takanashi et al. 2009). Recently, we reported that HP1β plays a tumor-promoting role in prostate cancer through transactivation of AR signaling as an AR coactivator that promotes prostate cancer cell proliferation (Shiota et al. 2010a). However, except for HP1β, the functions of the HP1 family in AR signaling and prostate cancer cell proliferation remain unknown. Additionally, the roles of each domain of HP1 in AR signaling and cancer cell proliferation remain unclear. Therefore, the aim of this study was to determine the functions of the HP1 family in association with the AR and prostate cancer cell proliferation.

Materials and methods

Cell culture

LNCaP and 22Rv1 human prostate cancer cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum. Passage 10–40 LNCaP cells were used in experiments. A castration-resistant derivative of LNCaP cells, LNCaP-CxR cells (referred to as CxR cells), was established and maintained as described previously (Shiota et al. 2010b). The cell lines were maintained at 37 °C with 5% CO₂.

Antibodies

Antibodies against the AR (sc-815) and green fluorescent protein (GFP) (sc-8334) were purchased from Santa Cruz Biotechnology. Anti-HP1α (#2623) and anti-HP1γ (#2619) antibodies were purchased from Cell Signaling Technology (Cambridge, MA, USA). Anti-HP1β (ab49938), anti-PSA, anti-β-actin, and anti-HA antibodies were purchased from Abcam (Cambridge, MA, USA), Epitomics (Burlingame, CA, USA), Sigma, and Roche Applied Science respectively.

Plasmid construction

The AR reporter plasmid (AR-Luc #3) was constructed as described previously (Shiota et al. 2010b). Construction of pCMV-HA-HP1α, pCMV-HA-HP1β, and pCMV-HA-HP1γ plasmids expressing N-terminal HA-tagged HP1α, HP1β, and HP1γ proteins respectively has been described previously (Shiota et al. 2010a). To prepare GFP-HP1α, GFP-HP1β, and GFP-HP1γ plasmids expressing N-terminal GFP-tagged HP1α, HP1β, and HP1γ proteins, respectively, EcoRI-Sall fragments of HP1α, HP1β, and HP1γ cDNAs from pCMV-HA-HP1α, pCMV-HA-HP1β, and pCMV-HA-HP1γ were inserted into the EcoRI-Sall sites of pEGFP plasmids (C3; Invitrogen) respectively. The pCMV-HA-HP1α ΔC (aa 1–116) plasmid expressing the N-terminal HA-tagged C-terminally deleted HP1β protein was created by deletion of the Xhol fragment from the pCMV-HA-HP1β plasmid. Other deletion mutants were constructed from GFP-HP1α, GFP-HP1β, GFP-HP1γ, and pCMV-HA-HP1β plasmids using a KOD Mutagenesis Kit (Toyobo, Osaka, Japan) with the following primer pairs: 5’-TCTCGAGTACCCGGATCCGGATGATATTG-3’ and 5’-ATGACTCTCTTCTTCTCTTTTTTATAG-3’ for GFP-HP1α ΔC (aa 1–112); 5’-ATCGCTCGGAGACTTTGAGAGGAGCGGATTATTG-3’ and 5’-AATTCGAAGCTTGAAGAGAGCGGATTATTG-3’ for GFP-HP1β ΔN (aa 113–190); 5’-GGTGATGGGAGGCCGGAGGGTTATTG-3’ and 5’-GTACGTGACGAGCTCTCTCCATGCG-3’ for GFP-HP1γ ΔN (aa 116–185); 5’-GTGGCATGGATGGGAGGCCGGAGGGTTATTG-3’ and 5’-AGCAGCATCTCTTCTCTTTCTTTATG-3’ for GFP-HP1γ ΔC (aa 1–110); 5’-GCTGAAAACCCAGAGGATTGGCCAG-3’ and 5’-AATTCGAAGCTTGAAGAGAGCGGATTATTG-3’ for pCMV-HA-HP1β ΔN (aa 116–185). The integrity of mutated plasmids was confirmed by sequencing. To construct pCMV-HA-HP1α ΔC (aa 1–112), pCMV-HA-HP1β ΔN (aa 113–190), pCMV-HA-HP1γ ΔC (aa 1–110), and pCMV-HA-HP1γ ΔN (aa 111–182) plasmids, Xhol-Sall fragments from GFP-HP1α ΔC, GFP-HP1β ΔN, GFP-HP1γ ΔC, and GFP-HP1γ ΔN were inserted into Xhol-Sall sites of pCMV-HA plasmids respectively.

Western blotting

The preparation of whole cell extracts and western blotting were performed as described previously (Shiota et al. 2010a,b, 2011a,b).

Knockdown analysis using siRNA

Knockdown analysis using siRNA was performed as described previously (Shiota et al. 2010a,b, 2011a,b). Briefly, the following double-stranded RNA oligonucleotides were commercially generated: 5’-CAU- AUCCUGAGGAUGCGGATT-3’ (sense) and 5’-UCCGCAUCCUCAGGAUGUUGTT-3’ (antisense) for HP1α siRNA (Sigma); 5’-AUUCUACAGGAACUGACUCUC-3’ (sense) and 5’-GGAGACUGCUAGUCCUGAU- GAAAU-3’ (antisense) for HP1β siRNA (Invitrogen); and 5’-CCAAGAGGAUUUGCAGACTGTT-3’ (sense) and
5′-CUCUGGCAAUCUCUCUGGTT-3′ (antisense) for HP1γ siRNA (Sigma). LNCaP and CxR cells were transfected with the indicated amounts of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Luciferase reporter assay, RNA isolation, RT-PCR and quantitative real-time PCR, cell proliferation assay, and flow cytometry

These procedures were performed as described previously (Shiota et al. 2010a,b, 2011a,b).

Statistical analysis

A t-test was used for statistical analysis. P values <0.05 were considered to be statistically significant.

Results

HP1γ knockdown transcriptionally reduces AR expression

First, we functionally analyzed AR expression by knockdown using HP1-specific siRNAs in LNCaP prostate cancer cells. HP1β has been shown to affect PSA expression but not AR expression as an AR coactivator in our previous study (Shiota et al. 2010a). Similarly, HP1α knockdown exerted no effect on AR expression. On the other hand, HP1γ silencing reduced the AR transcript level (Fig. 1A). Consistently, AR protein expression was also suppressed by HP1γ silencing (Fig. 1B), and also major AR-target gene PSA expression was suppressed by HP1β and HP1γ knockdown. In addition, using an AR reporter plasmid (AR-Luc #3) in a luciferase reporter assay, we found reduced luciferase activity by HP1γ silencing, indicating that HP1γ knockdown downregulates AR transcription (Fig. 1C).

Figure 2

HP1γ silencing reduces prostate cancer cell proliferation by inducing cell cycle arrest at G1 phase. (A) LNCaP cells were transfected with 40 nM of the indicated siRNA and then cultured. The number of cells was counted at the indicated times. The results were normalized to the number of cells at 0 h. All values represent at least three independent experiments. Boxes, mean; bars, ± s.d. *P<0.05 (compared with control siRNA). (B) LNCaP cells were transfected with 40 nM of the indicated siRNA and then cultured. At 72 h post-transfection, cells were stained with propidium iodide and analyzed by flow cytometry. The cell cycle distribution is shown in the upper right of each graph.

Figure 3

Expression of the HP1 family by expression plasmids. (A) Schematic representation of full-length and deletion-mutant HP1 isoforms. (B) Whole cell extracts of LNCaP cells transfected with 1 μg/ml of the indicated expression plasmid and then incubated for 48 h were subjected to western blotting using the indicated antibodies.
**HP1γ silencing reduces prostate cancer cell proliferation by inducing cell cycle arrest at G1 phase**

Next, we investigated the effects of HP1 isoforms on prostate cancer cell proliferation by HP1 knockdown. Previously, we showed that HP1β knockdown reduces prostate cancer proliferation by suppressing AR trans-activation (Shiota et al. 2010a). Here, we examined the effect of HP1α and HP1γ on cell proliferation. The data showed that HP1γ silencing reduced LNCaP cell proliferation, whereas HP1α knockdown did not affect cell growth (Fig. 2A). To reveal the mechanism of cell growth retardation by HP1γ silencing, we performed cell cycle analysis using flow cytometry. As shown in Fig. 2B, HP1γ suppression resulted in an accumulation of LNCaP cells in G1 phase, indicating cell growth suppression by inducing cell cycle arrest at G1 phase.

**Overexpression of HP1 affects AR expression and prostate cancer cell proliferation**

Next, we examined the effect of HP1 overexpression on AR expression and prostate cancer cell proliferation. To overexpress the HP1 family, we constructed HA-tagged HP1 expression plasmids, as well as deletion mutants to investigate the roles of the CD and CSD (Fig. 3A). The expression of HA-tagged full-length and deletion-mutant HP1 isoforms was confirmed by western blotting using whole cell extracts (Fig. 3B). Next, AR transcript levels were examined after overexpression of various HP1 isoforms. As shown in Fig. 4A, overexpression of HP1α and HP1α ΔC increased the AR transcript level, which was supported by the results of luciferase reporter assays using an AR reporter plasmid (Fig. 4B). Furthermore, cell proliferation was enhanced by overexpression of HP1β, which was in agreement with our previous report, as well as full-length HP1α and HP1α ΔC (Fig. 4C).

Furthermore, we examined the role of the HP1 family in prostate cancer cell proliferation using another AR-expressing prostate cancer cell line, 22Rv1. Expression of GFP-HP1 isoforms in 22Rv1 cells was confirmed by western blotting as shown in Fig. 5A. After overexpression
of the HP1 family, 22Rv1 cell proliferation was augmented by HP1β and more strongly by HP1α (Fig. 5B).

**HP1 expression and function in CRPC**

To investigate the relevance of the HP1 family in CRPC, we compared the expression of HP1 isoforms between LNCaP cells and its castration-resistant derivative, CxR cells. We found that HP1β expression was elevated in CxR cells, as well HP1β knockdown resulted in decreases of PSA expression and CxR cell proliferation (Shiota et al. 2010a). In addition, HP1α was upregulated, while HP1γ expression was comparable in CxR cells (Fig. 6A). Next, we examined the effect of HP1 knockdown on AR expression. As shown in Fig. 6B, AR expression was also reduced in CxR cells by HP1γ silencing, which was similar to that in LNCaP cells (Fig. 6B). As a result, HP1γ knockdown also suppressed CxR cell proliferation (Fig. 6C).

**Discussion**

Classically, the HP1 family has been thought to have suppressive effects on cancer growth and progression. For example, it has been reported that HP1α expression is reduced in invasive human breast cancer cell lines compared with that in non-invasive breast cancer cell lines (Kirschmann et al. 1999). Overexpression of HP1α in invasive cells reduces their in vitro invasive potential (Kirschmann et al. 2000), whereas reducing its expression increases their invasive potential (Norwood et al. 2006). These data suggest that HP1α acts as a progression suppressor in breast cancer cells. In addition, reduction of HP1α expression has been observed in metastatic colon cancer cell lines compared with that in non-metastatic cell lines (Ruginis et al. 2006), and in papillary thyroid...
However, recent findings suggest a conceptual shift from a suppressive to promoting role of the HP1 family in cancers. For example, HP1γ has been shown to be positive in all cancer tissues but is not positive in the matching normal epithelium (Takanashi et al. 2009). Consistently, suppression of HP1γ expression restrains the proliferation of various cancer cell types, but not that of normal cells, suggesting that HP1γ is a promising therapeutic target (Takanashi et al. 2009). Similarly, HP1α expression is upregulated in various tumor tissues in contrast to the corresponding normal tissues without detectable HP1α expression, as well as in highly malignant breast cancers with poor patient survival (De Koning et al. 2009). In addition, HP1-related methyltransferase G9a has been reported to promote lung cancer invasion and metastasis (Chen et al. 2010). In addition, we have recently shown that HP1β is upregulated with an increased Gleason score in prostate cancer (Shiota et al. 2010a).

Takanashi et al. (2009) reported that re-expression of HP1γ by overexpression maintains human pre-adipocytes in an undifferentiated state even with HP1γ suppression by a differentiation inducer, suggesting a role of HP1γ re-expression in maintaining the undifferentiated state of cells. Similarly, the HP1 family has been shown to be downregulated in differentiated blood lymphocytes compared with that in their undifferentiated precursors (Gilbert et al. 2003, Baxter et al. 2004, Ritou et al. 2007). Taken together, these results indicate that loss of HP1γ expression is essential for differentiation, suggesting a function as a safety mechanism for the transition to cellular differentiation. Consistently, our results showed that HP1γ knockdown suppressed prostate cancer cell growth, whereas overexpression of HP1γ showed no significant effect on cell proliferation. This result indicates that HP1γ overexpression is unnecessary for cell proliferation at least in our experimental setting, although it is necessary to maintain cell cycling.

By contrast, HP1α overexpression promoted prostate cancer cell proliferation, whereas HP1α silencing exerted no apparent suppressive effect on cell growth, which is consistent with a previous report showing that HP1α knockdown does not affect cell proliferation (Norwood et al. 2006) or cell cycle distribution (De Koning et al. 2009). Furthermore, the proliferation-promoting role of HP1α is consistent with previous findings indicating that oncogenic E2F (Weinmann et al. 2002, Oberley et al. 2003) and myc (Li et al. 2003, Kim et al. 2008) transcription factors positively regulate HP1α expression. These results appear to indicate a role of HP1α in cell proliferation as a growth factor, which can be substituted by other factors.

In addition to the tumor-promoting role of HP1 proteins, we revealed a role of HP1 isoforms in AR singling. We previously reported the role of HP1β as an AR coactivator in prostate cancer. In addition, we revealed the role of HP1α and HP1γ in AR expression: HP1α overexpression induced AR expression while HP1γ knockdown reduced AR expression, by overexpression and knockdown methods respectively. Although these findings were not recapitulated by reversing the expression of HP1α and HP1γ, they were correlated with cell proliferation, which was consistent with the role of the AR in cell proliferation of prostate cancer. As HP1β knockdown did not affect cell proliferation in AR-negative PC-3 cells (Shiota et al. 2010a), AR signaling may mediate the effect on cell proliferation by HP1, especially HP1β.

Overexpression of CD- or CSD-deleted HP1γ partially exerted a similar effect as that of full-length HP1γ, indicating that both the CD and CSD of HP1γ can retain partial activity of full-length HP1γ (Takanashi et al. 2009). Similarly, our study showed that HP1α ΔC exerted similar effects on AR signaling and prostate cancer cell proliferation as those of full-length HP1α, suggesting a supporting or compensating role of the CD of HP1α for full-length HP1α.

Intriguingly, HP1 isoforms are thought to be implicated in the DNA damage response induced by various toxins including oxidative stress (Dinant & Luijsterburg 2009). HP1 is recruited to damaged regions of DNA and facilitates the DNA repair pathway activated by oxidative lesions (Zarebski et al. 2009). These findings are interesting because it has been shown that oxidative stress is induced by androgen deprivation (Shiota et al. 2010b) and is increased in CRPC (Shigemura et al. 2007), which is consistent with upregulation of HP1α and HP1β in CRPC cells and their promoting role in AR signaling and cell proliferation in prostate cancer. These results indicate that HP1 isoforms participate in cross talk between oxidative stress and AR signaling in prostate cancer. Moreover, HP1 isoforms may augment therapeutic effects by genotoxic anticancer therapy that exerts oxidative stress, which is supported by the finding that a higher sensitivity to ionizing radiation is also observed in HP1α- or HP1β-overexpressing cells (Sharma et al. 2003).

In conclusion, HP1 isoforms distinctly promote AR signaling and prostate cancer growth, which may
contribute to progression to CRPC. Therefore, silencing of HP1β and HP1γ appears to be a promising therapeutic strategy for treatment of prostate cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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