The Role of Breast Carcinoma Amplified Sequences 2 as a Transcription Cofactor of Androgen Receptor (AR) via AR Protein Stabilization

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Abstract

We have found a novel gene, breast carcinoma amplified sequence 2 (BCAS2), by using androgen receptor (AR) as bait in a yeast-two hybrid screening system. BCAS2 has been previously reported to be a coactivator of estrogen receptor (ER). To explore the BCAS2 function in androgen-dependent prostate cancer, we found that BCAS2 can bind to AR in the presence of its cognate ligand (DHT) that enhances AR-driven prostate specific antigen (PSA) promoter activity that PSA is biosensor to monitor prostate cancer. Together with the other studies, it indicates that BCAS2 is also a coactivator of AR except ER. To further explore the mechanism of BCAS2 activating AR-driven gene expression, we find that BCAS2 can rescue AR protein degradation induced by geldanamycin, a HSP90 inhibitor, in LNCaP cells, regardless of nuclear and cytosol AR. Additionally, BCAS2 is significantly expressed in prostate cancer patients. In sum, BCAS2 can stabilize AR protein to enhance AR-mediated gene expression, and can also be diagnosis marker of prostate cancer that may be a critical target for developing therapeutic agents against prostate cancers.

Introduction

Prostate cancer is the most common type of cancer found in males in developed contries. It is the sixth leading cause of cancer death in males, accounting for 14% (903,500) of total new cancer cases and 6% (258,400) of the total cancer deaths in males of worldwide in 2008 (1). Androgen deprivation has been the standard therapy for advanced and metastatic prostate cancer for over half a century, as prostate tumors are initially dependent on androgens for growth and survival (2). Since the significance of androgens in the development of prostate cancer has been known, a lot of efforts have been made to study the significance of the specific nuclear receptor of the hormone, androgen receptor (AR) (1). The AR belongs to the superfamily of nuclear receptors that mediates the actions of steroids, retinoids, vitamin D3 and thyroid hormones (3). These receptors have similar structures that are composed of an N-terminal domain (NTD) that is involved in transcriptional activation, a DNA-binding domain, a hinge region and a ligand-binding domain. AR is a ligand-activated transcription factor that mediates the biological responses of androgens. After the ligand binds to the AR, the ligand–receptor complex translocates to the nucleus and binds specific androgen response elements (AREs) on the chromosome. The AR might regulate the expression of various genes (4). Most prostate cancers are androgen-dependent and respond to the anti-androgens or androgen-deprivation therapy. However, the progression to an androgen-independent stage occurs frequently. Possible mechanisms that could be involved in the development of hormone resistant prostate cancer causes including androgen receptor (AR) mutations, AR amplification/over expression, interaction between AR and other growth factors, and enhanced signaling in a ligand-independent manner (5, 6, 7). Androgen receptor gene amplification has been suggested as one of the molecular mechanisms responsible for the development of hormone refractory prostate cancer (6). HSP90 plays an important role in AR transcriptional activity (8). Studies on the effects of HSP90 inhibitor on AR functions in LNCaP prostate cancer cell line found that its administration led to decreased receptor stability, decreased associated gene transcription and an increased AR proteasomal degradation (9). HSP90 inhibitors have been developed as anticancer drugs (10,11). 17-AAG, one of the HSP90 inhibitors, was the first to enter into clinical trials (12).

We used AR as bait in a yeast-two hybrid screening system and found that Breast carcinoma amplified sequence 2 (BCAS2) is one interacting protein with AR. The BCAS2 gene maps to chromosome 1p13.3-21 region and contains 678 bp encoding 225 amino acids, with a predicted molecular mass of 26 kDa (13). BCAS2 was recently characterized as a transcriptional cofactor that enhances estrogen receptor–mediated gene expression (14). BCAS2 also be found associates with the tumor suppressor p53 protein. BCAS2 reduces p53 function. It mildly but consistently decreases p53 protein in normal condition. However, in the presence of DNA damage, BCAS2 prominently reduces p53 protein and provides protection against chemotherapeutic agent such as doxorubicin (15).

In our study, we found that BCAS2 can bind to AR in the presence of its cognate ligand (DHT) that enhances AR-driven prostate specific antigen (PSA) promoter activity. It indicates that BCAS2 is a coactivator of AR. When overexpression BCAS2 in LNCaP cells, stability of AR protein was increased. To further explore the mechanism of BCAS2 activating AR-driven gene expression, we find that BCAS2 can rescue AR protein degradation induced by HSP90 inhibitor in LNCaP cells. Additionally, BCAS2 is significantly expressed in prostate cancer patients. These results suggests that BCAS2 can stabilize AR protein to enhance AR-mediated gene expression, and can also be a diagnosis marker of prostate cancer that may be a critical target for developing therapeutic agents against prostate cancers.

Materials and Methods

Cell culture and transfection. 293T cells were cultured in DMEM medium with 10% fetal bovine serum (FBS). Transfection was performed by calcium phosphate/DNA co-precipitation procedure, which was modified by standard procedures (16). LNCaP cells were cultures in RPMI 1640 medium supplemented with 10% FBS. Transfection of LNCaP cells was performed by electroporation method by BTX ElectroSquarePorator™ ECM830 apparatus (Harvard Bioscience Company, USA) according to procedures that manufacturers provided on BTX website (http://www.btxonline.com/).

Plasmids. The expression plasmids for C-terminally Flag-tagged BCAS2 has been constructed by polymerase chain reaction (PCR) using MCF-7 cDNA as template and cloned into p3XFLAG-CMV14 (Sigma, USA) vector. The expression vector pGEX-4T-1 (GE Healthcare Life Sciences, USA) vector was generated by tagging GST at N-terminally of BCAS2 by PCR method performed as described previously (17). The pSUPER (OligoEngine, USA) vector-mediated RNAi system for shBCAS2 was constructed according to manufacturers' protocol.

Antibodies. Antibodies used were: anti-BCAS2 (Bethyl Labortories, USA), anti-AR (Santa Cruz Biotechnology, USA); Anti-actin and anti-FLAG (M2) antibodies were from Sigma (USA).

Yeast Two-hybrid Screen A pACT2-HeLa MATCHMAKER cDNA library (Clontech) that consists of the GAL4 activation domain (aa 768–881) fused with a human HeLa cDNA library was transformed into CG-1945 yeast strain (Clontech), along with a plasmid, pAS2–1-$AR_{595–918}$, containing GAL4 DBD (aa 1–147) fused with the C-terminal domain of AR (aa 595–918). $\sim 5 \times 10^6$ yeast transformants were screened and selected on synthetic dropout (S.D.; Difico) medium lacking leucine, tryptophan, and histidine in the presence of 25 mM 3-amino-1,2,4-triazole (3-AT; Sigma) and 10 nM dihydrotestosterone (DHT; Sigma). Colonies were tested for LacZ reporter gene activity in a β-galactosidase filter assay. Plasmid DNAs from positive clones were recovered from yeast, amplified in *Escherichia coli*, and confirmed by sequencing.

GST fusion proteins pull-down and In vitro transcription/translation pull-down analysis. The GST and GST fusion protein plasmids were transformed into *E. coli* B21 (DE3) strain. These fusion proteins were purified by glutathione-Sepharose 4B beads (GE Healthcare Life Sciences, USA) according to procedures that manufacturers provided. For GST pull-down analysis, equal volumes of GST fusion proteins immobilized on beads (10 μl) were incubated with nuclear lysates of LNCaP cells (10 mg). The proteins bound on beads were analyzed by SDS-PAGE and Western blot analysis. In vitro transcription/translation were performed by used The TNT® Quick Coupled Transcription/Translation System (Promega, USA) according to manufacturers' instructions. The *in vitro* translated AR was incubated with the immobilized GST or GST fused proteins beads. The bound proteins were subject to run 10% SDS-PAGE followed by Western blotting analysis.

Nuclear/Cytoplasm fraction. Nuclear and cytoplasm fractions were prepared by resuspending trypsinized cells in cold buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2.5 mM DTT (Dithiothreitol), 0.2 mM PMSF (Phenylmethylsulphonyl fluoride), and 1X protease inhibitor (Merck, Germany) by gentle vortexing, followed by incubation on ice for 10 min. After centrifugation the supernatants were transferred into new tube (cytoplasm fraction). The pellet was washed three times with buffer A. The pellet was then suspended with buffer C (20 mM HEPES-KOH pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 25 % glycerol, 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA, and 1X protease inhibitor), mixed well by pipetting, followed by incubated on ice for 20 min. After centrifugation, transferred the supernatant into new tube (nuclear fraction). Both fractions were processed for immunoblot analysis.

Immunofluorescence microscopy. Cells were fixed with 4% paraformadehyde., then double stained with anti-AR and anti-BCAS2, followed by incubation with FITC-conjugated anti-rabbit (Abcom, UK) and Alexa Fluor-conjugated anti-mouse antibodies (Invitrogen, USA). Nucleus signal was stain with DAPI. Fluorescent images were monitored by Leica confocal microscope.

Co-immunoprecipitation analysis. To determine BCAS2-AR binding, LNCaP cell lysed was prepared for immunoprecipitation. Cells were lysed in lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP40, 1 mM PMSF, and 1X protease inhibitor), centrifuged for 5 min at 12,000*g* and the insoluble debris was discarded. Cell lysate (500 μg protein) was immunoprecipitated using anti-BCAS2 or anti-AR antibody and protein A–agarose beads for 3 hours at 4 °C. The beads were washed with lysis buffer three times, then boiled in SDS sample buffer, The bound proteins were subject to run 10% SDS-PAGE followed by Western blotting analysis by anti-BCAS2 or anti-AR antibodies.

Luciferase activity assays. Transient transfections were performed with electroporation in LNCaP cells. 48 hours after tansfection, the cells were harvested and the luciferase activity was assayed using a Dual-Glo ^{TM} Luciferase Assay System (Promega, USA) according to the manufacture's instruction, then detected by Luminoskan Ascent luminometer (Thermo, USA). The results of the promoter firefly luciferase activities were normalized by internal control *Renilla* luciferase activities (pRL-CMV).

Cell-cycle and apoptosis analysis. The transfected cells in 6-cm plates were trypsinizd and collected. After fixed by ice-cold 70% ethanol cells were stained with propidium iodide and analysed by flow cytometry (BD FACSCalibur, USA). The WinMDI software was used for data analysis.

Cell proliferation assay. LNCaP cells were transfected by electroporation method. 24 hours after transfection, the cells were harvested by trypsinization. LNCaP cells were re-seeded at 2 x 10^4 per well in 24-well culture plates. The total cell numbers were counted at 24, 48, 72, 96 hour using hemocytometer by trypan blue staining.

RNA extraction and Semi-quantitative RT-PCR. Total cells RNA was extracted by using the TRIzol Reagents (Invitrogen, USA) as the manufacturer's instructions. Semi-quantitative RT–PCR analysis was performed using total RNA as described (17,18).

Prostate cancer specimens and Immunohistochemistry. Prostate Cancer specimens were obtained from National Taiwan University Hospital and Prostate cancer tissue array (PR953) from US Biomax Incorporation. Immunohistochemistry stain of prostate cancer slides were performed by used The Super Sensitive Polymer-HRP IHC Detection System (BioGenex, USA) according to manufacturers' protocol. Briefly, paraffin slides were deparaffinized in xylene and rehydrated in a graded series of ethanol concentrations. Slides were reacted with human BCAS2 polyclonal antibody (1:500 dilution; Protein Tech Group, USA) and counterstaining with hematoxylin. The scores of BCAS2 expression are based on the following two parameters-one for the intensity scores from 0 to 3 [0 (faint brown nuclear staining); 1+ (intermediate brown nuclear staining); 2+ (brown nuclear staining), and 3+ (dark brown nuclear staining); second for the range from 0 to 100% expression proportion of the field. The score was counted by intensity X range. High: score higher or equal 150; low: lower than 150 (19). All samples were scored by two pathologists.

SRB assay. The SRB assay was performed using a method modified from National Cancer Institute (20). Briefly, shRNA infected or non-infected cells were seeded in 96-well plates ($3X10^4$ cells/well) in medium with 5% serum. After 24 h, cells were fixed with 10% trichloroacetic acid (TCA) to represent cell population at the time of drug addition (T_0). After additional incubation of the drug or solvent for 48 h, cells were fixed with 10% TCA and then stained with SRB at 0.4% (w/v) in 1% acetic acid. Washed by 1% acetic acid to remove unbound SRB and then SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at wavelength of 515 nm by ELISA reader. Using the following absorbance measurements, such as time zero (T_0), control growth (C), and cell growth in the presence of the drug (T_x), the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as: $100 - [(T_x - T_0)/(C - T_0)] \times 100$. Growth inhibition of 50% (IC_{50}) is determined at the drug concentration which results in 50% reduction of total protein increase in control cells during the drug incubation.

Statistical analysis. Statistical procedures were carried out using SPSS 16.0 software. Pearson' chi-square test was done. P values < 0.05 were considered significant.

Results

BCAS2 and AR physically interact in prostate cancer cells.

In this study, we isolated *BCAS2* by using the androgen receptor (amino acids 595–918) as the bait to screen a human HeLa MATCHMAKER cDNA library in a yeast two-hybrid screening assay. To further characterize the associated between AR and BCAS2, the nuclear extracts of LNCaP cells was performed by GST pull-down assay. Immunoblotting confirmed that AR was bound to GST-BCAS2 fusion protein (Figure 1A). We then asked whether BCAS2 could directly interact with AR. The AR protein synthesized by *in vitro* transcription/translation was incubated with the various purified GST fusion proteins in GST pull-down assay. The results showed that the AR protein could be pulled down with GST-BCAS2 proteins (Figure 1B). These data indicate that BCAS2 directly interacted with AR. When investigating the interaction between endogenous BCAS2 and AR in LNCaP prostate cancer cells, AR protein could be detected by immunoprecipitate use anti-BCAS2 antibody (Figure 1C, panel a). BCAS2 protein also could be precipitated by AR antibody (Figure 1C, panel b). We also conducted Immunofluorescence assay and observed by confocal microscopy. The results showed endogenous BCAS2 and AR are colocalized inside the LNCaP nucleus (Figure 1D). In summary, our data showed that BCAS2 could directly interact with AR *in vitro* and *in vivo*.

Overexpression of BCAS2 increases transcriptional activity of liganded AR in prostate cancer cells.

The AR is a ligand-activated transcription factor that can activate downstream target genes. After the interactions between BCAS2 and AR have been identified, we further investigated whether BCAS2 affected AR transcriptional activity. Prostate specific antigen (PSA) is a well-known AR target gene. Transcription of the PSA gene was enhanced by AR binding to its promoter region, which contains androgen response element (ARE) sites (21, 22, 23). Firstly, we co-transfected the PSA promoter-Luciferase plasmid along with p3XFLAG-BCAS2 and internal control pRL-CMV into 293T cells as an assay model to determine AR transcriptional activity (17, 18). The total amount of plasmid per dish was equalized by adding empty vectors. The results showed that the luciferase activity of the PSA promoter was increased with BCAS2 protein over-expression (Figure 2A). Next we knockdown BCAS2 expression in LNCaP cells, and the results showed that abolished BCAS2 expression would reduced PSA promoter luciferase activity (Figure 2B). The results suggest that BCAS2 can enhance AR transcriptional activity.

BCAS2 enhances AR mRNA and protein levels in prostate cancer cells.

It has been reported that p53 could repress AR gene expression. The regulation mechanism was concluded at transcriptional level (24). We also found BCAS2 could reduce p53 function (15). Using RT-PCR, we observed that ectopic expression or knockdown BCAS2 expression in LNCaP cells, the AR and its downstream gene PSA mRNA would increase or decrease along with BCAS2 expression level (Figure 3A). It shows that BCAS2 could enhance AR mRNA level. Transfected pSG5-AR plasmid, which was control by SV40 promoter, into 293T cells, we found that AR protein level would increase follow BCAS2 expression level (Figure 3B). Ectopic expression of BCAS2 in LNCaP cells also enhances AR protein level regardless ligand exist (Figure 6A). The results suggest that BCAS2 influence not only AR mRNA but also protein level. We further to explore the role of BCAS2 on AR protein expression. The effect of BCAS2 overexpression on AR protein stability was evaluated using cycloheximide (CHX), which inhibits protein synthesis by blocking ribosomes. We observed that ectopic expression of BCAS2 would enhance AR protein stability regardless ligand treatment in LNCaP cells (Figure 3C, panel a and b). In summary, these findings indicate that BCAS2 could enhance AR mRNA and protein levels in prostate cancer cells.

BCAS2 expression is associated with prostate cancer.

To characterize levels of BCAS2 expression in prostate cancer patients, we analyzed 83 human prostate cancer tissues collected from National Taiwan University Hospital and prostate cancer tissue array from commercial by immunohistochemistry. Figure 4A and 4B illustrates that BCAS2 expression is low in normal prostate tissue but high in prostate cancer tissue. BCAS2 expression is significantly more frequent (*, $P < 0.05$) in prostate cancer tissues compared with normal prostate tissues (Figure 4C).

BCAS2 knockdown inhibits prostate cancer cell proliferation and induces apoptosis.

To determine the biological effect of knockdown BCAS2 gene expression in LNCaP cells. LNCaP cells were transfected with the pSUPER, shBCAS2 and non-function shRNA (shCon) vectors separately to determine the cell growth rates. The results shows that cell growth were significant reduction after treatment with shBCAS2 both in ligand with (Figure 5A, panel a) or without (Figure 5A, panel b) conditions. To assess effect of BCAS2 knockdown on cell apoptosis, we evaluated its effect on cell-cycle population using flow cytometry. As shown in Figure 5B, the fraction of cells undergoing apoptosis (sub-G1) was significantly increased in BCAS2 shRNA treated LNCaP cells. The results suggest that BCAS2 could influence prostate cancer cell growth and survival.

BCAS2 knockdown increases the cellular sensitivity to HSP90 inhibitor.

Heat shock protein 90 (HSP90) is an essential molecular chaperone that functions on the correct folding and stabilization of various proteins in cells. During the last years, HSP90 has considers as a target for cancer therapy (25). Geldanamycin (GM) is a natural product that binds to conserved pocket of HSP90 protein (26, 27, 28). Previous study found that occupancy of HSP90 pocket by GM causes the degradation of several signaling proteins important in mediating prostate cancer growth. One of these is AR (29, 30,31). Treatment of prostate cancer cells with GM derivative 17-AAG also results in the degradation of AR (32). In our study, we transfected BCAS2 into LNCaP cells, then treated with GM. We found that BCAS2 could rescue AR protein degradation induced by HSP90 inhibitor in LNCaP cells (Figure 6A). To further analyzed which part's AR been rescued. We harvested nuclear and cytosol fraction proteins separately. The results show that BCAS2 could rescue both nuclear and cytosol AR protein degradation induced by GM in LNCaP cells (Figure 6B). Owing to HSP90 inhibitor could degrade AR, decrease BCAS2 also cause AR degradation. We next investigated whether silencing BCAS2 in combination with pharmacological HSP90 inhibitor 17-AAG was synergistic. Following evaluated by SRB assay showed a significant decrease in IC_{50} value with the shBCAS2 compared with all controls (Figure 6C). The results suggest that depletion of BCAS2 would increase the cellular sensitivity to HSP90 inhibitor 17-AAG.

Figure Legends

Figure 1. Interaction between BCAS2 and AR *in vitro* and *in vivo*.

A, BCAS2 associates with AR *in vitro*. GST-BCAS2 and GST proteins were incubated with LNCaP nuclear extracts and analyzed by silver stained (top). GST-pull-down proteins were analyzed by Western blot (WB) with AR antibody (bottom). **B**, BCAS2 directly interacts with AR *in vitro.* The *In vitro* transcription/translation AR protein incubated with the GST, GST-BCAS2. The bound proteins were analyzed by Western blot using an anti-AR antibody and GST proteins were stained by coomassie blue. **C**, AR and BCAS2 reciprocal interaction *in vivo*. LNCaP nuclear extracts was immunoprecipitated with anti-BCAS2 **(a)** or anti-AR **(b)** antibody. Bound proteins were subjected to Western blot analysis. **D**, cellular localization of BCAS2 and AR by confocal laser microscopy. Bar, 10 μm.

Figure 2. BCAS2 enhances AR-driven PSA promoter activity.

A, Overexpression of BCAS2 increases AR-dependent transcription activity. 293T cells were transiently transfected with PSA-Luc promoter plasmid along with Flag-BCAS2 or pSG5-AR expression construct in the absence or presence of DHT (10 nM). **B**, Depletion of BCAS2 decreases AR-dependent transcription activity. LNCaP cells were transiently transfected with PSA-Luc promoter plasmid along with increasing doses of pSUPER-shBCAS2 plasmid, in the absence or presence of DHT (10 nM). All luciferase assays were done three times as independent experiments. Mean ± SD.

Figure 3. BCAS2 affects AR mRNA and protein levels.

A, BCAS2 expression affects AR mRNA level. LNCaP cells were transfected with shBCAS2 or Flag-BCAS2 plasmid. 24hrs after transfection cells were treated with DHT (10nM) or solvent for 24hrs. Cells RNA were extracted and subjected to RT-PCR analysis. **B**, BCAS2 expression increases AR protein amount. 293T cells were transiently transfected with pSG5-AR along with increasing doses of Flag-BCAS2 plasmid. 24 hour after transfection, cells were treated with DHT (10 nM) or solvent for 24 hrs. Cells lysates were harvested and subjected to WB analysis. **C**, BCAS2 enhances AR protein stability. LNCaP cells were transfected with Flag-BCAS2 or Flag-vector plasmid. 24hrs after transfection cells were treated with DHT (10 nM) or solvent for 24hr. 48 hour after transfection, cells were incubated with CHX (20 μg/ml) for the indicated time period. The amounts of AR protein from the lysates of cells in the presence **(a)** or absence **(b)** of DHT were analyzed by western blotting. The % of AR indicates the AR amount at each time point relative to the control (time 0 set as 100%).

Figure 4. BCAS2 was significantly overexpressed in prostate cancer specimens.

A, Immunohistochemical analysis of expression of BCAS2 in normal prostate and prostate cancer tissues. BCAS2 protein expression was measured by immunohistochemistry using anti human BCAS2 antibody. From left to right were Normal part from lesion tissue, Gleason Score ≤ 6, Gleason Score = 7, and Gleason Score ≥ 8 sections, all magnification, 200 X. **B**, BCAS2 expression in normal part paired with tumor lesions. **C**, BCAS2 is significantly overexpressed in prostate cancer specimens. The scores of BCAS2 expression are based on the following two parameters-one for the intensity scores from 0 to 3 [0 (faint brown nuclear staining); 1+ (intermediate brown nuclear staining); 2+ (brown nuclear staining), and 3+ (dark brown nuclear staining); second for the range from 0 to 100% expression proportion of the field. The score was counted by intensity X range. High: score higher or equal 150; low: lower than 150. The statistics analysis is used *P:pearson's chi-square test.

Figure 5. Depletion of BCAS2 decreases cell growth and induces apoptosis regardless of DHT.

A, Reducing the expression of BCAS2 increases apoptosis in LNCaP cells. shBCAS2 inhibits the growth rate of LNCaP cells in the presence (**a**) or absence (**b**) of ligand. Cell proliferation assays were performed. All experiments were performed three times with triplicate samples. The results show the mean of all data. Error bars indicate standard deviations. **B**, shBCAS2 induced apoptosis by flow cytometry analysis.

Figure 6. BCAS2 affects AR protein stability and cell sensitivity of HSP90 inhibitor.

A, BCAS2 can prevent AR protein degradation induced by geldamamycin (GA). LNCaP cells were transfected with Flag-BCAS2 or Flag-vector. 24 hrs after transfection, cells were treated with GA (3.5 μM) or solvent in the presence or absence of DHT (10 nM) as indicated for 24 hrs. Cells lysates were then collected and subjected to WB analysis for AR, Flag (BCAS2). **B,** BCAS2 rescues nuclear and cytosol AR protein degradation induced by GA as described in panel **A**, cells were harvested for nuclear/cytosol fractionation. Fraction samples were subjected to WB analysis for AR, Flag (BCAS2), HSP90, PARP (as nuclear fraction control) and α-tubulin (cytosolic fraction). Numbers below the panels of the immunoblotted indicate the densitometeric values normalized to the relative β-actin value (**A**) or PARP and α-tubulin value (**B**). **C**, BCAS2 knockdown increases the cellular sensitivity to HSP90 inhibitor. LNCaP cells were infected with lentiviral shBCAS2 or lentiviral shCon. 48 hour after infection, 3 X 10^4 cells per well were seeded onto 96-well plates and allowed to grow for 24 hour prior to different dosage 17-AAG treatments for a further 48 hour. Cell proliferation was then measured by SRB assay.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
2. Golias Ch, Iliadis I, Peschos D, Charalabopoulos K. Amplification and co-regulators of androgen receptor gene in prostate cancer. *Exp Oncol.* 2009;31:3-8.
3. Burnstein KL. Regulation of androgen receptor levels: implications for prostate cancer progression and therapy. *J Cell Biochem*. 2005;95:657-69.
4. Zilliacus J, Wright AP, Carlstedt-Duke J, Gustafsson JA. Structural determinants of DNA-binding specificity by steroid receptors. *Mol Endocrinol*. 1995;9:389-400.
5. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, Trapman J. Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int J Cancer*. 1991;48:189-93.
6. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat Genet*. 1995;9:401-6.
7. Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Res*. 1999;59:2511-5.
8. Georget V, Térouanne B, Nicolas JC, Sultan C. Mechanism of antiandrogen action: key role of hsp90 in conformational change and transcriptional activity of the androgen receptor. *Biochemistry*. 2002;41:11824-31.
9. Vanaja DK, Mitchell SH, Toft DO, Young CY. Effect of geldanamycin on androgen receptor function and stability. *Cell Stress Chaperones*. 2002;7:55-64.
10. McDonald E, Workman P, Jones K. Inhibitors of the HSP90 molecular chaperone: attacking the master regulator in cancer. *Curr Top Med Chem*. 2006;6:1091-107.
11. Smith, J.R.; Workman, P. Targeting the cancer chaperone HSP90. *Drug Discov Today Ther Strateg.* 2006; 4:219-227
12. Pacey S, Banerji U, Judson I, Workman P. Hsp90 inhibitors in the clinic. *Handb Exp Pharmacol*. 2006;172:331-58.
13. Nagasaki K, Maass N, Manabe T, et al. Identification of a novel gene, DAM1, amplified at chromosome 1p13.3-21 region in human breast cancer cell lines. *Cancer Lett.* 1999;140:219-26.
14. Qi C, Zhu YT, Chang J, Yeldandi AV, Rao MS, Zhu YJ. Potentiation of estrogen receptor transcriptional activity by breast cancer amplified sequence 2. *Biochem Biophys Res Commun.* 2005;328:393-8.
15. Kuo PC, Tsao YP, Chang HW, Chen PH, Huang CW, Lin ST, Weng YT, Tsai TC, Shieh SY, Chen SL. Breast cancer amplified sequence 2, a novel negative regulator of the p53 tumor suppressor. *Cancer Res*. 2009;69:8877-85
16. Wigler M, Pellicer A, Silverstein S, Axel R. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell*. 1978;14:725-31.
17. Chen PH, Tsao YP, Wang CC, Chen SL. Nuclear receptor interaction protein, a coactivator of androgen receptors (AR), is regulated by AR and Sp1 to feed forward and activate its own gene expression through AR protein stability. *Nucleic Acids Res.* 2008;36:51-66.
18. Tsai TC, Lee YL, Hsiao WC, Tsao YP, Chen SL. NRIP, a novel nuclear receptor

interaction protein, enhances the transcriptional activity of nuclear receptors. *J Biol Chem*. 2005;280:20000-9.

19. Han CP, Lee MY, Tzeng SL, Yao CC, Wang PH, Cheng YW, Chen SL, Wu TS, Tyan YS, Kok LF. Nuclear Receptor Interaction Protein (NRIP) expression assay using human tissue microarray and immunohistochemistry technology confirming nuclear localization. *J Exp Clin Cancer Res*. 2008;27:25.
20. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst*. 1990;82:1107-12.
21. Cleutjens KB, van der Korput HA, van Eekelen CC, van Rooij HC, Faber PW, Trapman J. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol Endocrinol*. 1997;11:148-61.
22. Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO, Trapman J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J Biol Chem*. 1996;271:6379-88.
23. Huang W, Shostak Y, Tarr P, Sawyers C, Carey M. Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. *J Biol Chem*. 1999;274:25756-68.
24. Alimirah F, Panchanathan R, Chen J, Zhang X, Ho SM, Choubey D. Expression of androgen receptor is negatively regulated by p53. *Neoplasia*. 2007;9:1152-9.
25. Cheung CH, Chen HH, Cheng LT, Lyu KW, Kanwar JR, Chang JY. Targeting Hsp90 with small molecule inhibitors induces the over-expression of the anti-apoptotic molecule, survivin, in human A549, HONE-1 and HT-29 cancer cells. *Mol Cancer*. 2010;9:77.
26. Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, Krutzsch H, Ochel HJ, Schulte TW, Sausville E, Neckers LM, Toft DO. The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J Biol Chem*. 1997;272:23843-50.
27. Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell*. 1997;89:239-50.
28. Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell*. 1997;90:65-75.
29. Schneider C, Sepp-Lorenzino L, Nimmesgern E, Ouerfelli O, Danishefsky S, Rosen N, Hartl FU. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc Natl Acad Sci U S A*. 1996;93:14536-41.
30. Mimnaugh EG, Chavany C, Neckers L. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *J Biol Chem*. 1996;271:22796-801.
31. Whitesell L, Cook P. Stable and specific binding of heat shock protein 90 by geldanamycin disrupts glucocorticoid receptor function in intact cells. *Mol Endocrinol*. 1996;10:705-12.
32. Solit DB, Zheng FF, Drobnjak M, Münster PN, Higgins B, Verbel D, Heller G, Tong W, Cordon-Cardo C, Agus DB, Scher HI, Rosen N. 17-Allylamino-17-

demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clin Cancer Res*. 2002;8:986-93.

Figures

Figure 1.

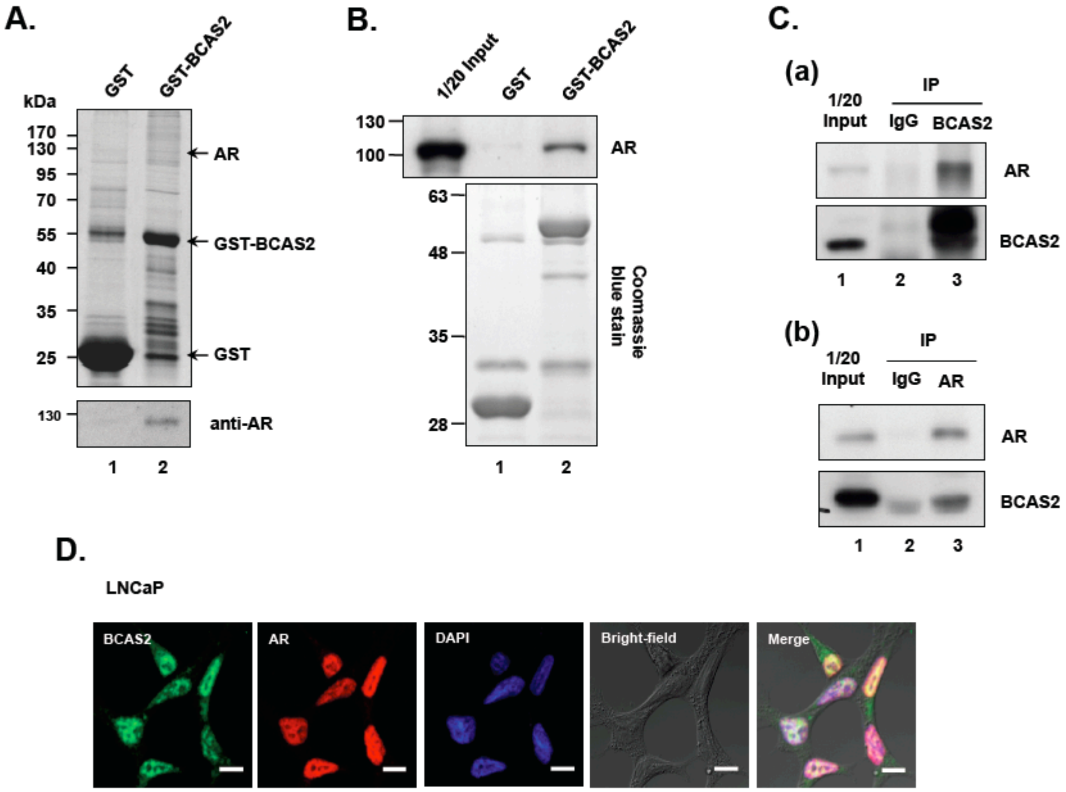


Figure 2.

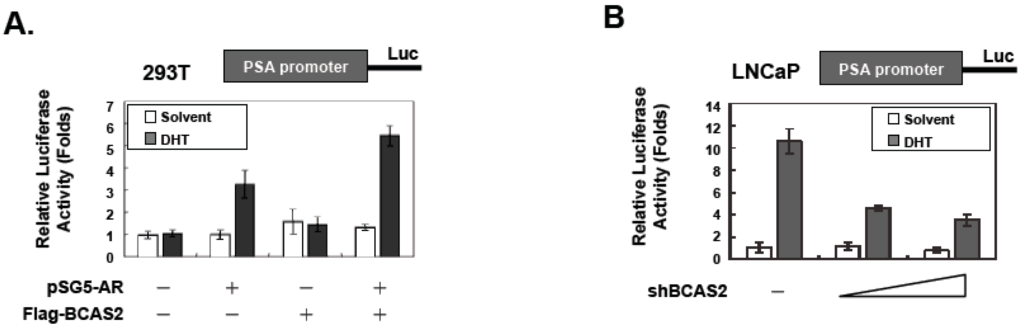


Figure 3.

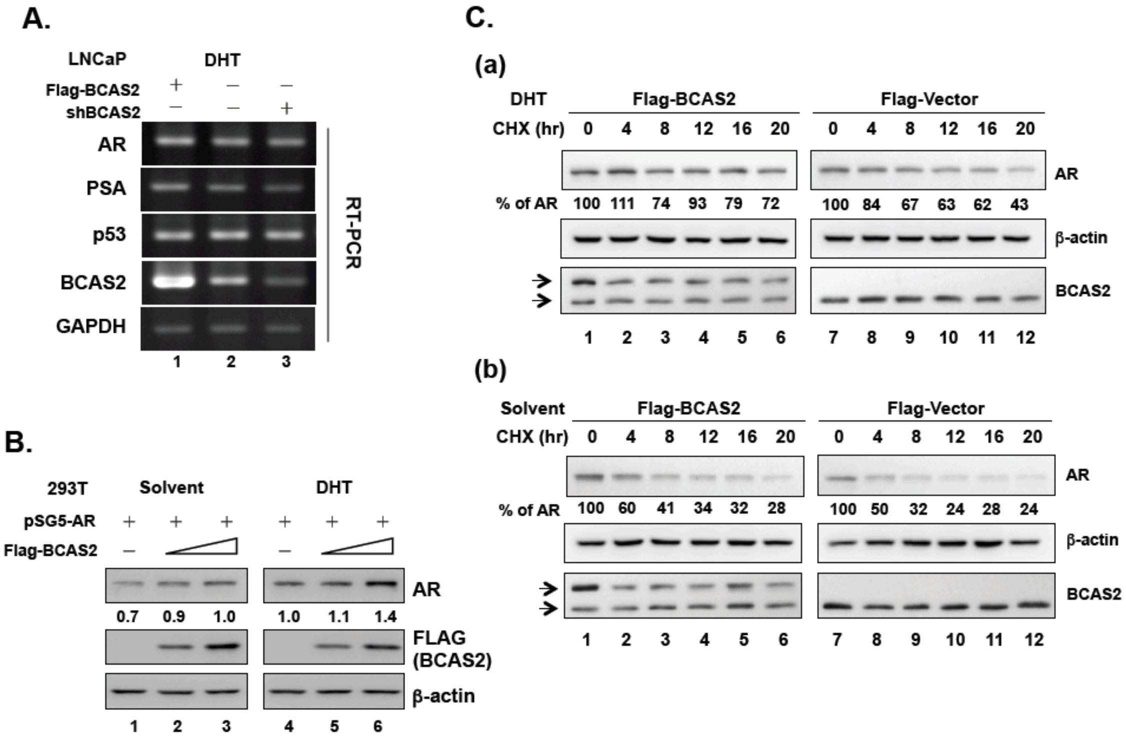
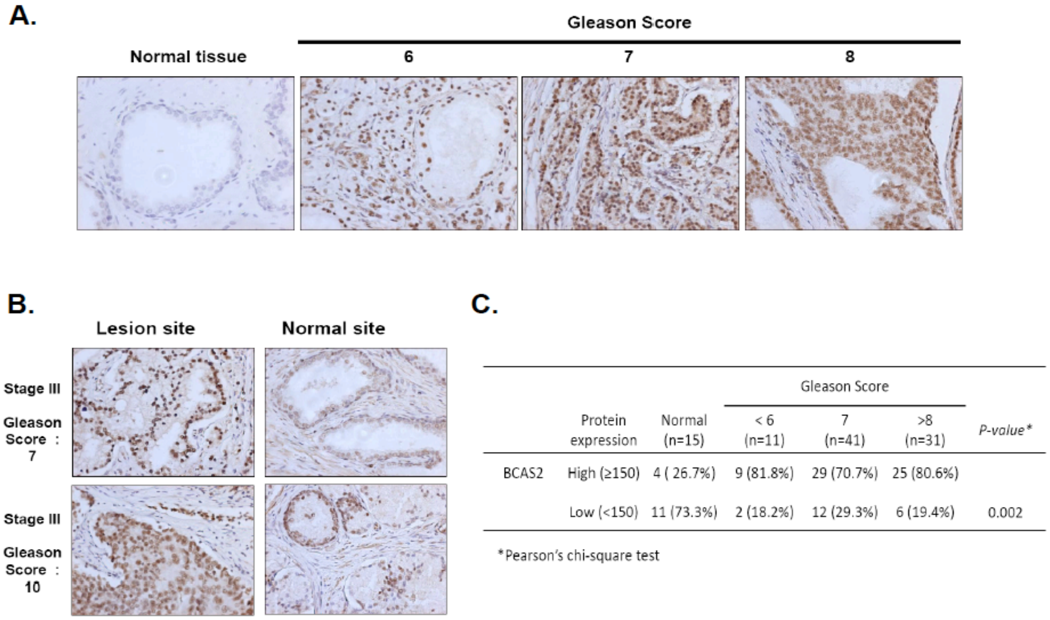


Figure 4.



	Protein expression	Normal (n=15)	Gleason Score < 6 (n=11)	7 (n=41)	>8 (n=31)	*P-value**
BCAS2	High (≥150)	4 (26.7%)	9 (81.8%)	29 (70.7%)	25 (80.6%)	
	Low (<150)	11 (73.3%)	2 (18.2%)	12 (29.3%)	6 (19.4%)	0.002

*Pearson's chi-square test

Figure 5.

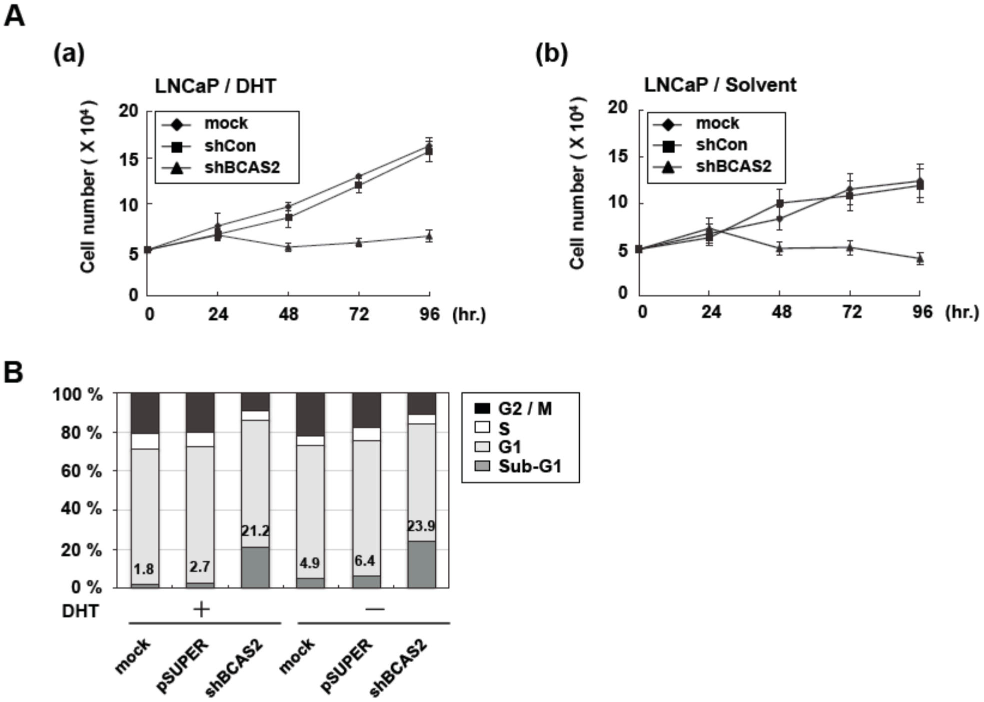


Figure 6.