Original Research

Bicalutamide enhances fodrin-mediated apoptosis through calpain in LNCaP

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Impact statement

We studied bicalutamide's anticancer action by using proteomics. The effect of bicalutamide on androgen-exposed LNCaP cells was also studied. KEGG identified >1.8-fold differentially expressed proteins between test group cells. KEGG mapper showed fodrin-mediated apoptosis involvement in bicalutamide's action. The anticancer effects of bicalutamide, which was further confirmed using Western blotting. Therefore, this drug is a potential candidate for understanding bicalutamide's effect on LNCaP and fodrin can be used as a biomarker monitoring status in metastatic carcinoma.

Abstract

Prostate cancer is the most common cancer in men, and before it progresses and metastasizes, the anticancer drug bicalutamide is often administered to patients. Many cases of androgen-dependent prostate cancer develop resistance during treatment with bicalutamide. Therefore, the effect of bicalutamide on androgen-dependent LNCaP prostate cancer cells is of clinical interest. The aim of this study was to demonstrate the effects of the anticancer drug bicalutamide on LNCaP prostate cancer cells by using a proteomics approach. Based on the results, 314 proteins were differentially expressed between the LNCaP and LNCaP treated with bicalutamide. The apoptosis pathway associated with differentially expressed proteins was shown in the Kyoto Encyclopedia of Gene and Genome pathway mapper. The Kyoto Encyclopedia of Gene and Genome pathway mapper results revealed that the fodrin-mediated apoptosis pathway is associated with the actions of bicalutamide and Western blotting was performed to validate these results.

Keywords: Apoptosis, bicalutamide, calpain, fodrin, LNCaP, proteomics

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Introduction

The incidence of prostate cancer, which is the most common cancer in men, is steadily increasing.[1] Because androgen serves as a growth factor in prostate cancer, the cells are more sensitive to androgen than normal cells and continue to grow during exposure to this hormone, which is one of the most important characteristics of prostate cancer.[2] To decrease the androgen effect, hormone therapy is used to treat prostate cancer.[2] Bicalutamide is known to inhibit the growth of prostate cancer by binding to the androgen receptor rather than androgen[3] and is typically used to treat patients with prostate cancer.

Before cancer metastasizes to other organs, hormone therapy with bicalutamide is administered.[4] The majority of progressive and metastatic prostate cancers have an 80 to 90% high response rate to hormone therapy.[5] However, after an average of 18–24 months, cancer often recurs, and the prostate biomarker prostate-specific antigen (PSA) is elevated again.[5] It has been reported that when bicalutamide is administered to patients with prostate cancer who do not respond to the male hormone, PSA is decreased by $\geq$ 50% in 23% of patients.[4] However, the duration of action was as short as 3–15 months.[6]

Furthermore, bicalutamide has been reported to have induced the apoptosis of prostate cancer cells.[7,8] It was reported that when bicalutamide was applied to LNCaP cells, apoptosis was induced by caspase 3.[9] In addition, proapoptotic proteins such as Bcl-2-associated X protein mediate apoptosis in androgen-independent PC3 cells.[9] The SAPK/JNK and MEK/ERK1/2 signaling pathways are involved in the inhibition of androgen-independent prostate cancer cell proliferation by bicalutamide.[10] However, previous studies targeted specific molecules or signals, and widespread alterations in proteins associated with apoptosis in prostate cancer have not been investigated yet. Therefore, our aim was to identify the alteration in

global protein expression related to apoptosis induced by bicalutamide treatment in prostate cancer cells. In particular, we designed the study to compare LNCaP and bicalutamide-treated LNCaP cells as well as RWPE-1 and LNCaP cells. The proteomics approach is suggested to be a potentially useful tool to exploit in the discovery of new apoptotic factors or apoptosis pathway associated with bicalutamide.[11]

To understand the mechanism of apoptosis pathway in prostate cancer, the patterns of protein expression were compared between the RWPE-1 and LNCaP. Additionally, we compared the LNCaP and LNCaP-Bic to demonstrate the apoptotic effects of bicalutamide on these cells. We envisage that research on the effect of bicalutamide on the apoptosis pathway in the LNCaP prostate cancer cell line would enhance the understanding of the processes involved in progressive and metastatic prostate cancer.

Materials and methods

Cell culture

Androgen-dependent LNCaP and RWPE-1 cells were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% carbon dioxide at 37°C at cell passages 26 and 32. Bicalutamide attenuates the growth effect of androgen in prostate cancer cells by competitively binding the androgen receptor. To evaluate alteration by competitive inhibition of bicalutamide, we compared LNCaP androgen-dependent prostate cancer cells with RWPE-1 cells as normal cells. Androgen was depleted in the LNCaP and RWPE-1 cells for 72 h. Next, androgen and bicalutamide were applied for 72 h. The cells that received bicalutamide received the two molecules at the same time.

Protein extraction and sample preparation and tryptic digestion

The cellular protein concentration was assessed using the Bradford assay (Bio-Rad, Hercules, CA, USA). LNCaP and RWPE-1 cell protein samples (1 mg) were reduced using 5 mM Tris(2-carboxyethyl)phosphine (Pierce, Rockford, IL, USA) for 30 min at 37°C. The samples were alkylated by treating them with 15 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) and then they were left for 1 h at 25°C rotated at 300 r/min in the dark. The samples were then treated with mass-spectrometry (MS) grade Trypsin Gold (Promega, Madison, WI, USA) overnight at 37°C. The chemical reagent was removed using C18 cartridges (Waters, Milford, MA, USA).

Peptide fractionation using OFFGEL electrophoresis

The tryptic peptide was applied to the OFFGEL Fractionator using a 12-well setup (3100 OFFGEL Low Res kit, pH 3–10; Agilent Technologies, Santa Clara, CA, USA), which separates peptides based on their isoelectric point. The protocol used was that provided in the manufacturer's instructions. Microspin columns (Harvard Apparatus, Holliston, MA, USA) were used for chemical reagent clearance.

Nano-liquid chromatography–tandem mass spectrometry

The peptide fraction was loaded into the high-performance liquid chromatography (HPLC)-chip/quadrupole time-of-flight (Q-TOF) system (Agilent Technologies, Santa Clara, CA, USA), which included an Agilent 6520 Q-TOF equipped with a chip cube interface and Agilent 1200 series nano-LC system. The samples were placed into a 360-nL enrichment column and a Polaris C18-A separation column (75 μm × 150 mm, 3 μm). The flow rate was 0.3 μL/min maintained for 120 min. The ionization mode of the ion source was positive, while the drying temperature and flow rate were 300°C and 3 L/min, respectively. The MS and MS/MS scan ranges were m/z 300–2400 and 100–3000 (at a rate of 4 spectra/s), respectively.

Protein identification using database search

The MS/MS spectra of the sample were exported and searched against an MS/MS database using the Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA, USA) to identify proteins and peptides. The search parameters were as follows: precursor mass tolerance, 20 ppm; product ion mass tolerance, 50 ppm; maximum ambiguous precursor charge, 3; two missed cleavages were allowed; fully digested peptide using trypsin; fixed modification of carbamidomethyl cysteine; variable modifications of oxidized methionine; and N-terminal carbamylation. The MS/MS spectra were autovalidated using a default value. The false discovery rate (FDR) threshold was 1.2.

Label-free quantification of protein expression

The relative quantification was carried out using the Mass Profiler Professional (MPP) software (Agilent Technologies, Santa Clara, CA, USA) with quantile normalization and filtering. Quantile normalized protein intensities were assessed to determine the relative amounts of proteins. Similarly, differentially expressed proteins with an expression difference of at least 1.8 fold were determined from normalized protein intensities. Statistical significance was evaluated by asymptotic *P*-value computation.

Functional analysis of altered protein

Bioinformatics analysis of the 1.8-fold differentially expressed proteins was performed using the Kyoto Encyclopedia of Gene and Genome (KEGG) enrichment analysis (http://cgap.nci.nih.gov/Pathways/) program. The apoptosis pathway was chosen in the KEGG category. Functional analysis of the 1.8-fold differentially expressed proteins was conducted using Metacore™ GeneGo software.

Western blot analysis

Proteins were extracted from the RWPE-1, LNCaP, and LNCaP-Bic cells and separated using sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. Antibodies against caspase 3 (Santa Cruz, sc-7272), calpain 1 large subunit (Cell Signaling, #2556), and Spectrin alpha chain (Fodrin, Millipore, #MAB1622) were used for the Western blotting. Western blot data were evaluated from three independent experiments.

Statistical analysis

The 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay results were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) software for Windows (version 16.0; SPSS Inc., Chicago, IL, USA). Independent *t*-test was used to determine statistical significance in Western blotting experiments performed in triplicate.

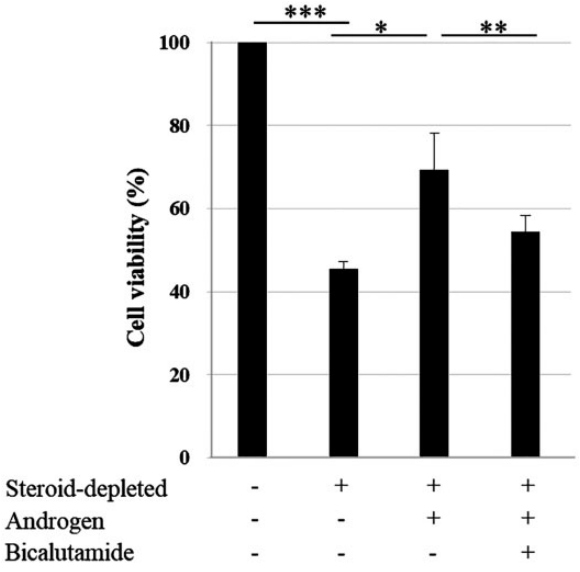

Figure 1. Viability of LNCaP and bicalutamide-treated LNCaP (LNCaP-Bic) cells in steroid-depleted medium. Cell viability was determined using XTT assay. Cell viability was decreased in steroid-depleted medium. Androgen treatment of LNCaP cells increased cell viability again and bicalutamide induced apoptosis of LNCaP cells. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Results

The RWPE-1 and LNCaP cells were treated with androgen, while the LNCaP-Bic cells were also treated with bicalutamide to determine the anticancer effects. LNCaP cell viability was altered in the androgen-depleted medium. Specifically, when the steroid hormone was depleted, the cell viability was 45.5%. The LNCaP cells were also treated with androgen, which increased their cell viability by 74.9%. However, bicalutamide induced apoptosis and decreased the cell viability by 49.4% again (Figure 1). We identified 1282, 837, and 1223 proteins in the RWPE-1, LNCaP, and LNCaP-Bic, respectively, while 41, 367, and 221 proteins were common to the RWPE-1 and LNCaP, RWPE-1 and LNCaP-Bic, LNCaP and LNCaP-Bic groups, respectively. Furthermore, 512 proteins were common to all three samples (Figure 2(a)), and 2641, 2510, and 2420 peptides were identified in the RWPE-1, LNCaP, and LNCaP-Bic groups, respectively. The results identified >2-fold differentially expressed proteins, while 347 and 314 proteins were differentially expressed between RWPE-1 and LNCaP and between LNCaP and LNCaP-Bic (Figure 2(b)).

In comparison with proteins identified only in the RWPE-1 group of cells, those identified only in the LNCaP were highly related to metabolic processes, cellular processes, and localization. Interestingly, although LNCaP cells not treated with bicalutamide showed upregulation of proteins related to metabolic processes, cellular processes, and localization, proteins of bicalutamide-treated LNCaP cells were downregulated similarly to those of RWPE-1 cells. The result of the analysis of molecular function showed that the percentage of proteins related to catalytic activity, binding, and structural molecule activity in RWPE-1 were 42%, 33%, and 13%, respectively (Figure 3(b)). In the LNCaP cells, catalytic activity, binding, and structural molecule activity were 51%, 31%, and 8% (Figure 3(b)).

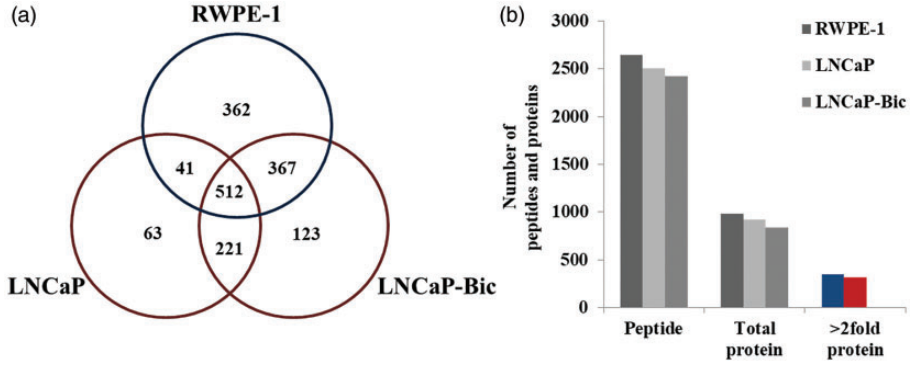

Figure 2. Identification of proteins in normal RWPE-1 prostrate and LNCaP prostate cancer cell lines were treated with androgen in steroid-depleted medium. LNCAP 2 cells were additionally treated with bicalutamide. (a) Venn diagram of proteins identified with at least two peptides in three groups. (b) Total number of peptides and proteins identified with at least two peptides in three groups and number of >1.8-fold differentially expressed protein. Red bar represents >1.8-fold differentially expressed protein in RWPE-1 vs. LNCaP. Blue bar represents >1.8-fold differentially expressed protein in LNCaP vs. LNCaP-Bic. (A color version of this figure is available in the online journal.)

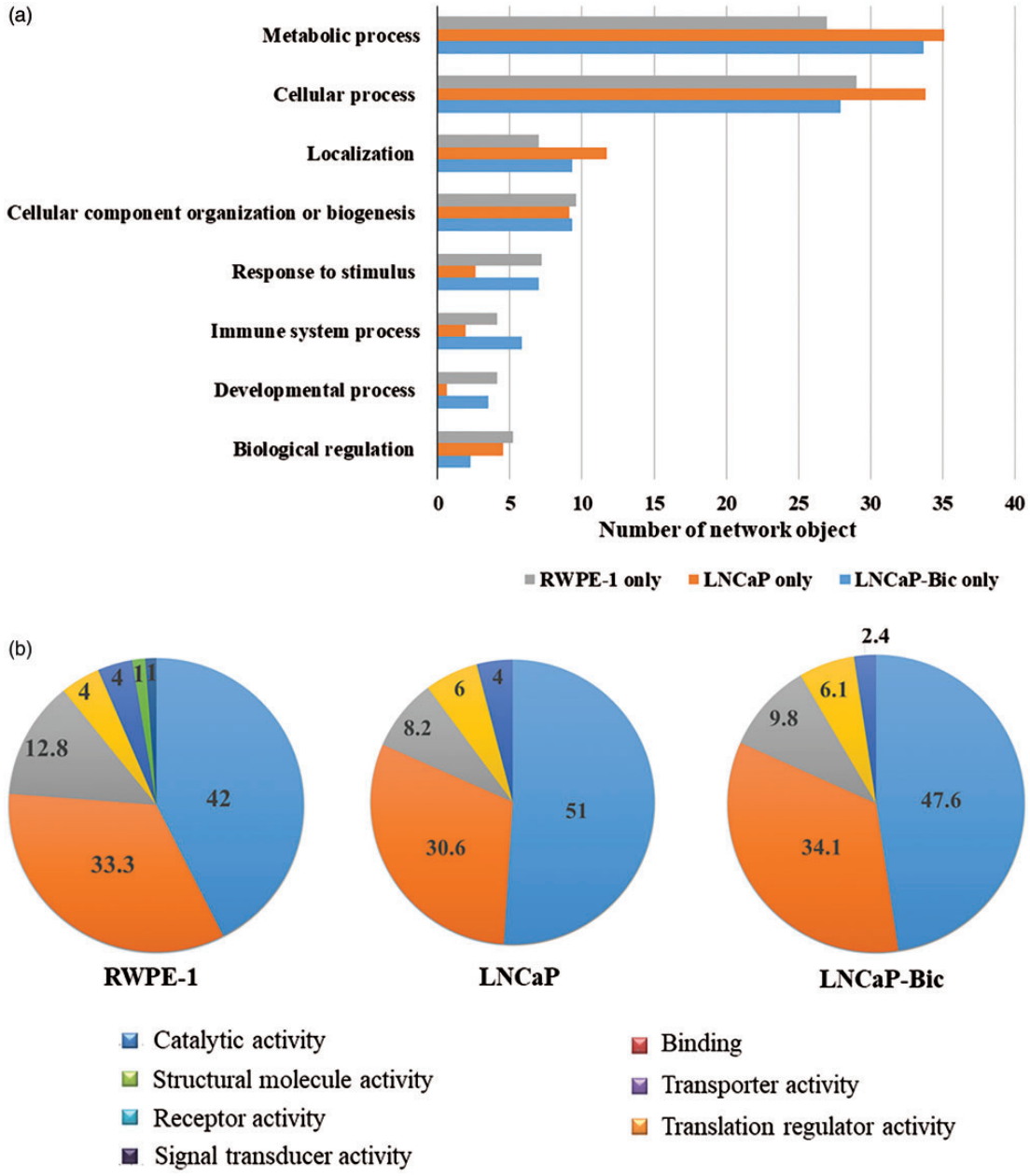

Figure 3. Functional analysis of identified proteins in RWPE-1, LNCaP and bicalutamide-treated LNCaP cells. (a)Biological process of RWPE-1, LNCaP, and LNCaP-Bic (b) Molecular function of RWPE-1, LNCaP, and LNCaP-Bic cells. *LNCaP-Bic, bicalutamide-treated LNCaP cells. (A color version of this figure is available in the online journal.)

Bicalutamide-treated LNCaP cells showed recovery of the changed molecular function compared with RWPE-1 cells.

The list of proteins differentially expressed by more than 1.8 fold in both RWPE-1 vs. LNCaP cells and LNCaP vs. LNCaP-bic cells was analyzed by the KEGG enrichment analysis (http://pantherdb.org/) mapper, and several pathways were identified including the apoptosis pathway. However, the importance of these pathways was different between RWPE-1 vs. LNCaP cells and LNCaP vs. LNCaP-bic cells. Because we tried to identify the pathways mediating the apoptosis induced by bicalutamide, we focused on the apoptosis pathway among several pathways and analyzed whether the expression pattern of apoptosis-related proteins was altered in LNCaP vs. LNCaP-Bic cells compared to RWPE-1 vs. LNCaP cells. Interestingly, although apoptosis-related proteins such as α-tubulin, actin, fodrin, lamin, and poly-ADP ribose polymerase (PARP) were downregulated in LNCaP cells compared to those in RWPE-1 cells, this pattern for fodrin was reversed after treatment with bicalutamide (Figures 4 and 5).

In the KEGG pathway, proteases such as those in the caspases family and calpain are activated by the tumor necrosis factor (TNF) and calcium signaling pathways. Activated proteases cleave substrates such as fodrin and actin, leading to decreased full-length substrates. In LNCaP cells compared with RWPE-1 cells, α-tubulin, actin, fodrin, lamin, and PARP, which are substrates of proteases, were downregulated (Figure 4). Among these

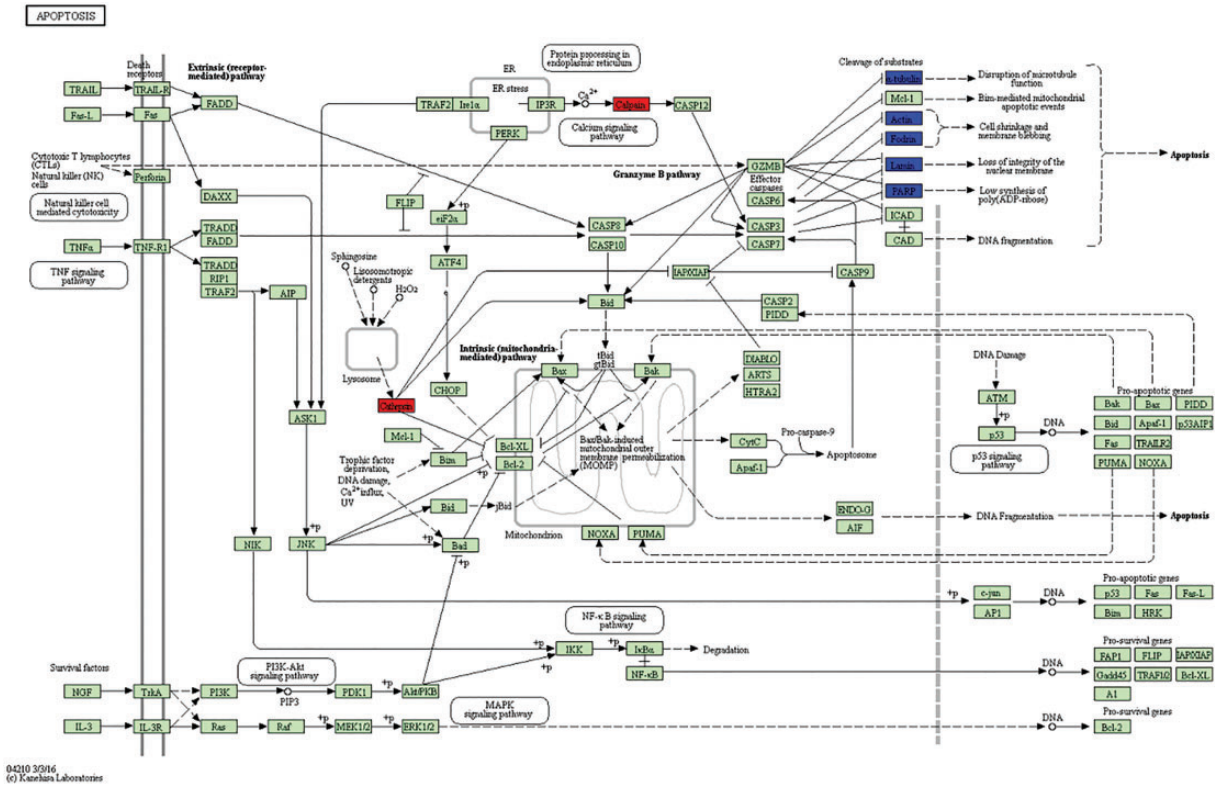

Figure 4. Apoptosis-associated pathway identified using Kyoto Encyclopedia of Gene and Genome (KEGG) mapper showing several differentially regulated proteins in normal RWPE-1 vs. LNCaP. Blue and red represent 1.8-fold downregulated and upregulated proteins, respectively, between RWPE-1 and LNCaP cells. (A color version of this figure is available in the online journal.)

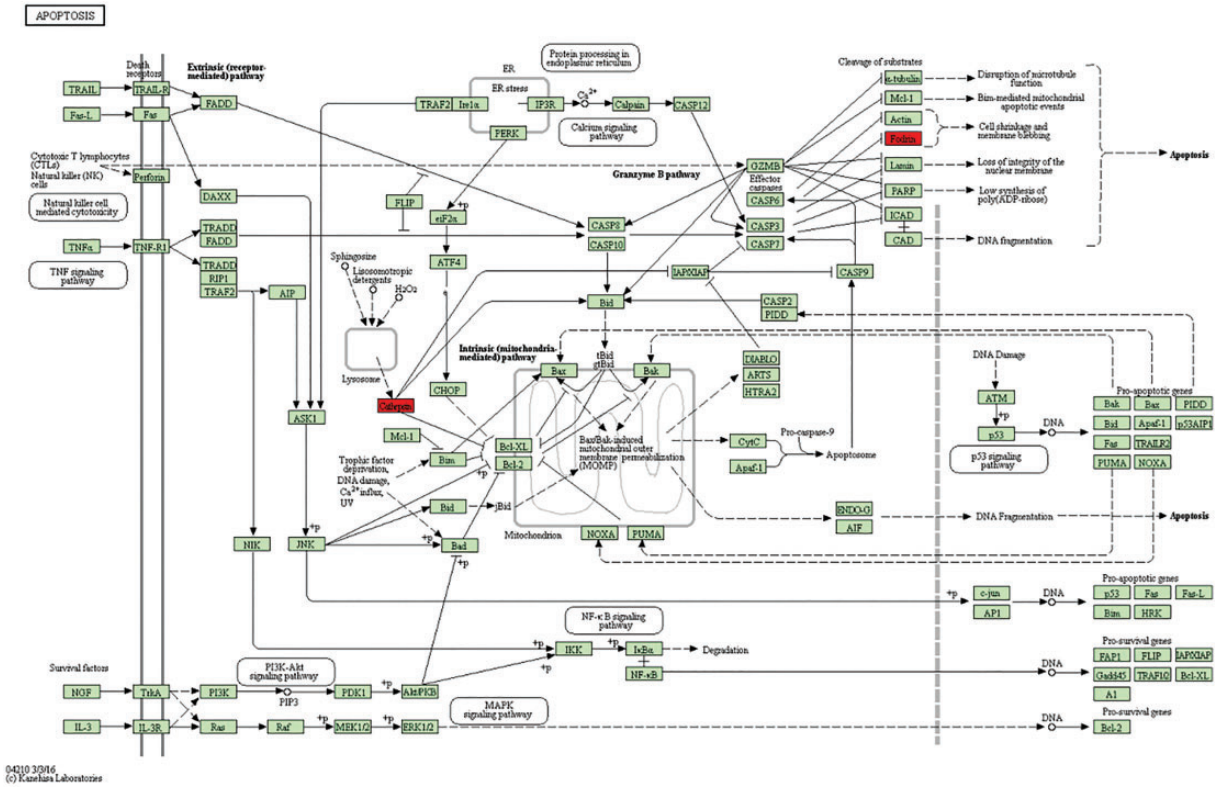

Figure 5. Apoptosis-associated pathway from Kyoto Encyclopedia of Gene and Genome (KEGG) mapper showing several differentially regulated proteins in LNCaP vs. bicalutamide-treated LNCaP cells. Red represents proteins downregulated or upregulated by at least 1.8-fold between LNCaP and LNCaP-Bic cells. (A color version of this figure is available in the online journal.)

(a)

50	MDPSGVKVLE	TAEDIQERRQ	QVLDRYHRFK	ELSTLRRQKL	EDSYRFQFFQ
100	RDAEELEKWI	QEKLQIASDE	NYKDPTNLQG	KLQKHQAFEA	EVQANSGAIV
480	---	K**LTVLSEER**A	ALLELWELRR	QQYEQCMDLQ	LFYRDTEQVD
1530	---	DKAINVQEEK	**IAALQAFADQ**	**LIAAGHYA**KG	DISSRRNEVL
2472	---	CVSHMKPYVD	GKGRELPTAF	DYVEFTRSLF	VN

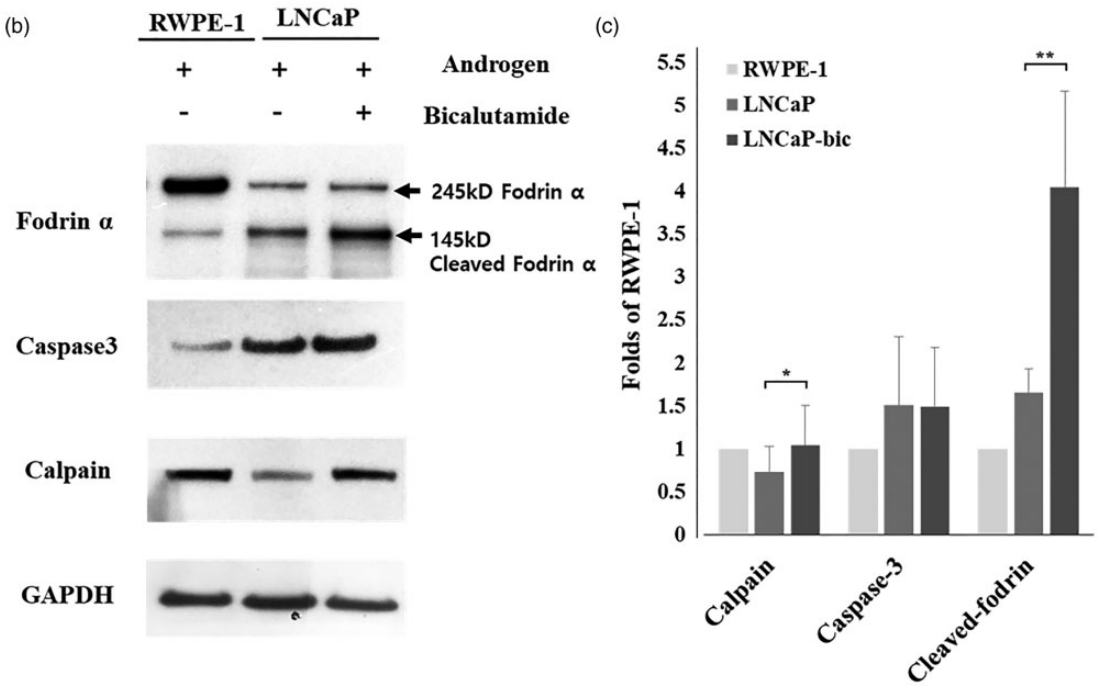

Figure 6. Identification of fodrin and actin using mass spectrometry (MS) and Western blotting. (a) Coverage of human fodrin sequence (in bold) identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). (b) Western blotting was performed using antibodies against caspase 3, calpain, and α-fodrin. Expression of full-length α-fodrin is higher than cleaved form in LNCaP. Cleaved fodrin was upregulated in bicalutamide-treated LNCaP cells compared to LNCaP cells without bicalutamide. Proteases of α-fodrin, caspase 3, and calpain were upregulated in LNCaP-Bibc compared to LNCaP cells. (c) Relativity protein expression to RWPE-1 was measured in LNCaP and LNCaP-bic. $^{*}P < 0.05$, $^{**}P < 0.01$ (independent *t*-test).

substrates, only fodrin showed a reversal of this pattern to upregulation when cells were treated with bicalutamide to block androgen (Figure 5). Because fodrin induces apoptosis through its cleavage, it was considered that the up-regulated fodrin in bicalutamide-treated LNCaP cells was the cleaved form of fodrin. However, because protein identification using LC-MS/MS is conducted using fragmented proteins, it cannot be determined whether the up-regulated fodrin observed in the KEGG pathway reflects full-length or cleaved fodrin (Figure 6(a)). Therefore, to determine whether or not the cleaved form of fodrin was up-regulated by bicalutamide, Western blotting was performed. To perform Western blotting, antibodies against spectrin (fodrin) and caspase 3 and calpain, which are known to cleave fodrin, were used together.

Fodrin is known to be cleaved by proteases such as caspase 3 and calpain, and Western blotting analysis showed that cleaved fodrin was up-regulated in LNCaP-Bic cells compared to LNCaP cells (Figure 6(b)). Based on the KEGG pathway and Western blotting results, it is suggested that bicalutamide mediates apoptosis through fodrin rather than other protease substrates such as α-tubulin, actin, lamin, and PARP. At the same time, calpain expression increased more in LNCaP-Bic cells than in LNCaP cells. Moreover, the expression in LNCaP cells was downregulated compared to that in RWPE-1 cells (Figure 6(b)). The protein expression relative to that in RWPE-1 cells was measured in LNCaP and LNCaP-bic cells (Figure 6(c)). In the LNCaP group, calpain-induced cleavage of fodrin was assumed to decrease compared to that in the RA group; however, treatment with bicalutamide re-activated the calpain-induced cleavage of fodrin. Additionally, protein–protein interaction (PPI) network analysis of differentially expressed proteins between LNCaP and LNCaP-Bic was performed using the Genego mapper. PPI demonstrates that α-fodrin is involved in the canonical pathway and is activated by calpain 1 and p54 (Figure 7).

Discussion

To demonstrate the efficacy of bicalutamide, the RWPE-1, LNCaP, and LNCaP-Bic groups were analyzed using a proteomics approach to identify global proteins.

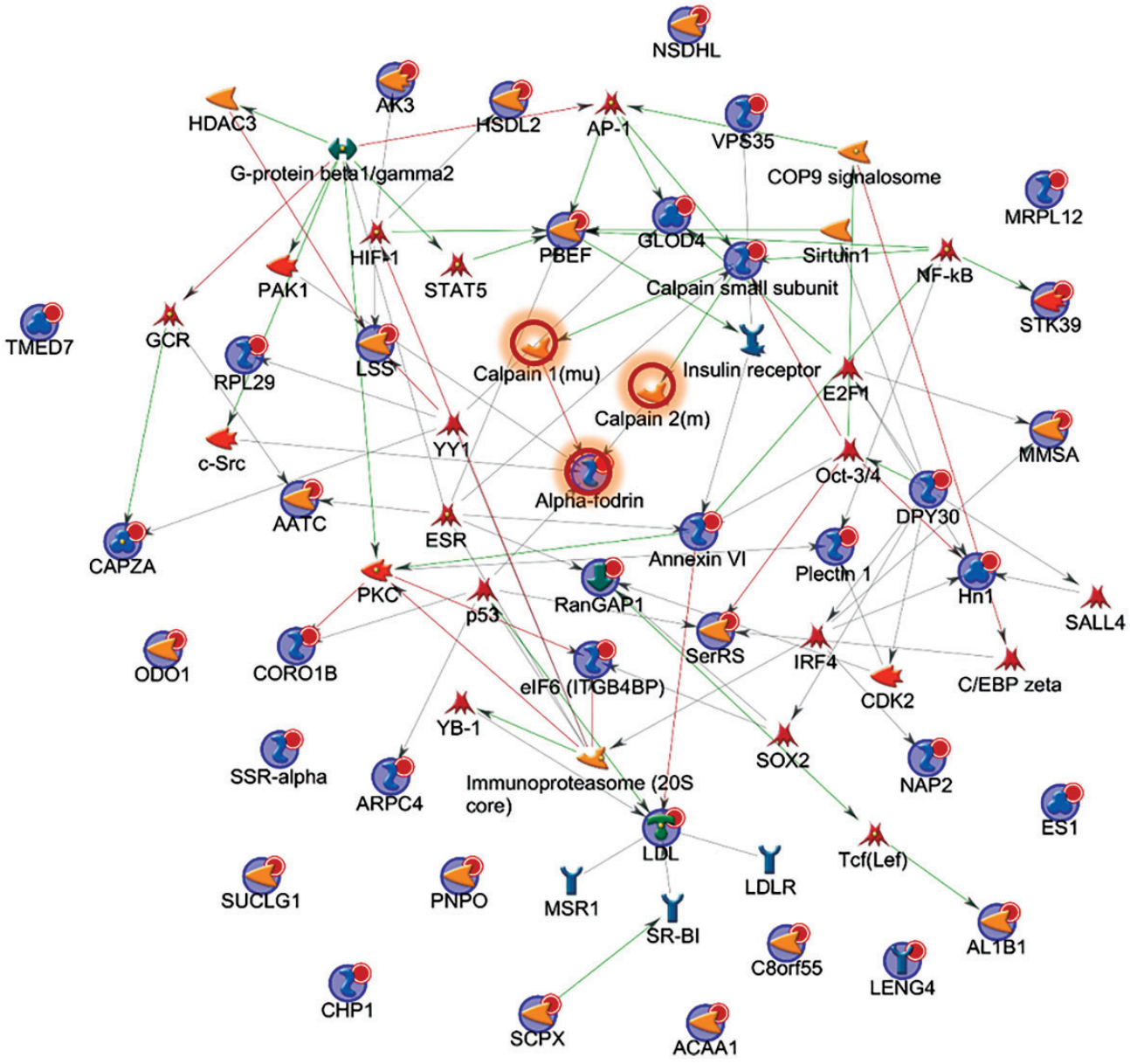

Figure 7. Protein–protein interaction (PPI) networks of differentially expressed proteins between LNCaP and bicalutamide-treated LNCaP. Line represents evidence of association. Blue line indicates canonical pathway described in Genego build network. Nodes are differentially expressed proteins. Red circle is α-fodrin and calpain 1. α-Fodrin is involved in the canonical pathway and activated by calpain 1. (A color version of this figure is available in the online journal.)

After bicalutamide treatment of LNCaP cells, the KEGG pathway analysis of differentially expressed proteins between the LNCaP-Bic and LNCaP group suggested that the fodrin-mediated apoptosis pathway is associated with calpain, and bicalutamide induces this pathway. The LNCaP prostate cancer cell line continues to grow when it is stimulated by androgen.[2] Our purpose was to demonstrate the apoptotic effect of bicalutamide on LNCaP cells by identifying the differentially expressed proteins between cells treated with and without bicalutamide and analyzing apoptosis pathways associated with the differentially expressed proteins. Before the LNCaP cells treated with and without bicalutamide were compared, they were both exposed to androgen in an androgen-depleted medium. This treatment was designed to preclude all the other effects including those of androgen to observe only the actions of bicalutamide.

Functional analysis was performed in RWPE-1, LNCaP, and LNCaP-Bic cells and the biological processes associated with metabolism and cellular processes increased in the LNCaP prostate cancer cells compared to the RWPE-1 cells. This observation suggests that prostate cancer cells activate metabolic and cellular processes to generate energy for cell proliferation and metastasis to other organs. Interestingly, in the LNCaP-Bic cells, biological processes altered in LNCaP cells compared to that in RWPE-1 cells were recovered to processes similar to those of RWPE-1 cells. Additionally, downregulation of the immune system

process in LNCaP cells in Figure 3(a) was reversed by bicalutamide to levels comparable to those of RWPE-1 cells. This result was consistent with previous results that immune system function was attenuated in cancer patients and recovered with anti-cancer drug treatment. This suggests that when an anti-cancer drug is administered, the cancer biological process recovers to a status similar to normal.

Fodrin is known to be cleaved by proteases such as calpain and caspase 3, and cleaved fodrin induces intrinsic apoptosis.[12] Cleaved fodrin translocates to the nucleus and mediates apoptosis.[13] Several studies have shown that apoptosis in cell lines is induced by the cleavage of fodrin.[14–17] Additionally, inhibition of caspase 3 and calpain has been shown to attenuate activation of fodrin and inhibit apoptosis.[18] Several studies have shown fodrin-mediated apoptosis by capase 3 and calpain in different cell lines.[19,20] In the KEGG pathway analysis, apoptotic substrates such as α-tubulin, actin, fodrin, lamin, and PARP that are targeted by proteases disrupt microtubule function, cell shrinkage, membrane blebbing, loss of integrity of the nuclear membrane, and low synthesis of polymerase and apoptosis. It is suggested that fodrin in the KEGG pathway reflects the full-length rather than cleaved fodrin. The Western blotting of fodrin to validate its involvement in apoptosis revealed that the expression of cleaved-fodrin was upregulated in LNCaP-Bic cells compared to the LNCaP cells, which suggests that bicalutamide induced fodrin-mediated apoptosis. While the expression of calpain decreased in the LNCaP cells, its expression subsequently increased in the LNCaP-Bic. Calpain is known to be activated by endoplasmic reticulum (ER) stress and induces mitochondrial-mediated apoptosis.[21] Additionally, calpain cleaves cytosolic fodrin, which then mediates apoptosis.[22] Therefore, bicalutamide is assumed to have an apoptotic effect on ER-stress via fodrin-mediated apoptosis in LNCaP cells. Fodrin-mediated apoptosis is reported to be induced by anticancer drugs in lung cancer A549 and HepG2 cells.[23] However, in A549 and HepG2 cells, fodrin is cleaved by caspase 3, and the induction of cleaved fodrin by calpain has not yet been reported. The fodrin-mediated apoptosis pathway induced by calpain activation in LNCaP-Bic is extremely interesting.

To demonstrate the efficacy of bicalutamide in LNCaP cells, a proteomics approach was used, and the results showed that differentially expressed proteins in LNCaP-Bic cells were associated with the fodrin-mediated apoptosis pathway. The Western blotting analysis suggests that fodrin was cleaved by calpain to induce the apoptosis of LNCaP-Bic cells. We demonstrated that while the expression of calpain and cleaved fodrin was downregulated in LNCaP cells, it was upregulated in LNCaP-Bic cells. The fodrin-mediated apoptosis pathway induced by bicalutamide could be a new potential target to be exploited for anticancer strategies. The difference in expression of apoptosis factors such as fodrin and calpain implies that these molecules could facilitate the understanding of the processes of metastatic carcinoma advancement during bicalutamide treatment in patients with non-metastatic carcinomas. Furthermore, the fodrin could be used as an early detectable biomarker of metastatic carcinoma.

Authors' contributions: JL wrote manuscript, analyzed data. SM wrote manuscript, generated figs/table. AP performed sample preparation. DK generated proteomic data BHC. HGK led the study, provided knowledge and expertise, critically reviewed manuscript. All authors read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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