Palmitoylome profiling indicates that androgens regulate the palmitoylation of α-tubulin in prostate cancer-derived LNCaP cells and supernatants

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Abstract. Prostate cancer is closely associated with constitutive transactivation of the androgen receptor (AR) signaling pathway. After treatment with androgen-deprivation therapy (ADT), the majority of patients develop castration-resistant prostate cancer within months or years. In order to investigate potential novel therapeutic targets in addition to ADT, the present study examined the regulatory mechanisms of the AR signaling pathway. In the present study, LNCaP cells were metabolically-labeled with Alk-C16, a palmitate probe. In addition, cells were treated with R1881, an androgen, or DMSO. Subsequently, click-chemistry-based palmitoylome profiling was performed in LNCaP cells and palmitoylated proteins were compared between cells treated with androgen and untreated cells. Androgen treatment was revealed to significantly increase the palmitoylation level of α-tubulin. In addition, the palmitoylation level of Ras-related protein Rab-7a (Rab7a) was enhanced by androgen treatment. Palmitoylation of α-tubulin and Rab7a were essential for cell proliferation. Notably, in the supernatant of LNCaP cells, the palmitoylation level of α-tubulin was also increased following androgen treatment. Palmitoylation of α-tubulin may provide a new potential target for the treatment of prostate cancer. In addition, the high level of α-tubulin palmitoylation in the supernatant may represent a biomarker for early-stage prostate cancer.

Introduction

Prostate cancer is the most common malignancy of the reproductive system in male patients, and is one of the leading causes of cancer-related mortalities in men worldwide. Prostate function is dependent on androgen (1). When the androgen receptor (AR) binds its ligands, including dihydrotestosterone, AR translocates into the nucleus, binds to androgen-responsive elements and regulates the expression of its downstream genes by interacting with various transcription factors. The AR signaling pathway is essential for the development, function and homeostasis of the prostate (2). However, the constitutive transactivation of the AR signaling pathway may result in prostate cancer initiation and progression. Although localized prostate cancer is treated with surgery or radiation (3), cancers at advanced and metastatic stages are typically treated using androgen-deprivation therapy (ADT) (4). However, the majority of patients may develop castration-resistant prostate cancer (CRPC) after months, or even years of ADT. At present, the progression of CRPC is believed to be caused by constitutive transactivation of the AR signaling pathway that may be dependent on low-levels of androgen or androgen-independent. Various activation mechanisms depend on AR activity and function, including AR amplification, AR mutations, AR splice variants and the abnormal activation of the AR (5). Therefore, numerous ADT agents have been identified over the years. However, AR activation mechanisms constantly evolve to antagonize these agents and may vary markedly among individuals. Therefore, in order to investigate novel therapeutic targets in addition to ADT, the mechanism underlying the AR signaling pathway requires further examination.

Protein fatty acid acylation is a newly identified co-translational or post-translational protein modification mechanism. This covalent attachment of lipids onto proteins can control protein-protein and protein-membrane interactions, thus serving important roles in human physiology and pathology. Protein palmitoylation is one form of fatty acid acylation and involves the covalent attachment of a 16-carbon fatty acid moiety, palmitate, onto amino acid residues (6). There are three types of palmitoylation, the occurrence of which depend on the type of bond formed between palmitate and amino acid residues: i) S-palmitoylation, presenting thioester bonds with cysteines; ii) O-palmitoylation, exhibiting ester bonds with serines or threonines; and iii) N-palmitoylation, forming amide bonds with lysines (7). There is a dynamic cycle between

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palmitoylated and non-palmitoylated protein forms. The regulation of this cycle depends on acyltransferases. In mammalian cells, S-palmitoylation, which is found in N-Ras and H-Ras, is catalyzed by a family of palmitoyltransferases with DHHC, or Asp-His-His-Cys, motifs, and is removed by fatty acyl protein thioesterases (8). By contrast, O-palmitoylation and N-palmitoylation are found on secretory proteins, including Wnt and Hedgehog, respectively (9). These palmitoylations are catalyzed by membrane-bound O-acyltransferases (10).

Palmitoylation promotes protein hydrophobicity, increasing membrane affinity, and alters the proteins subcellular localization, thus regulating protein trafficking, stability and activity (11). Accumulating evidence has indicated that many palmitoylated proteins are associated with tumorigenesis and metastasis in prostate cancer. For example, Src-family tyrosine kinases (SFKs) are involved in signaling pathways that regulate cell proliferation, survival, motility and adhesion (12). The efficient activity of SFKs, including FYN proto-oncogene, Src family tyrosine kinase, LYN proto-oncogene, Src family tyrosine kinase, YES proto-oncogene 1, Src family tyrosine kinase and HCK proto-oncogene, Src family tyrosine kinase, require association with the plasma membrane, and this association is mediated by palmitoylation (13). Neurotensin receptor 1 is a G-protein-coupled receptor that mediates cancer progression in prostate cancer. Palmitoylation is essential for the localization of certain proteins in specific membrane microdomains and for the activation of their downstream signaling pathways (14). Considering the key roles of palmitoylated proteins in tumorigenesis and the limitations of the currently available treatments for CRPC, the present study aimed to identify the androgen-induced palmitoylated proteins and examine potential novel regulatory mechanisms of the AR signaling pathway in order to develop novel therapies for prostate cancer.

Click-chemistry-based chemical probes for the detection of protein palmitoylation may allow the rapid discovery of novel palmitoylated proteins and the understanding of their biological functions (15). The combination of mass spectrometry (MS)-based quantitative proteomics with click-chemistry-probes can be used to examine the dynamics of protein palmitoylation under different physiological or pathological conditions (16,17). In the present study, chemical tools were used, and palmitoylated proteins between androgen-treated LNCaP cells and untreated LNCaP cells were investigated using palmitoylome profiles. The present study aimed to identify candidate proteins exhibiting palmitoylation levels that could be promoted by androgen treatment. The regulation of the palmitoylation of candidate proteins may provide new directions for the development of novel therapies to treat prostate cancer. Moreover, the palmitoylation of specific proteins induced by androgen treatment may represent a novel biomarker for prostate cancer.

Materials and methods

Cell culture. LNCaP and PC3 cells were purchased from ATCC and cultured in RPMI-1640 medium supplemented with 10% FBS. RWPE-1 cells were provided by The Stem Cell Bank, Chinese Academy of Sciences, and were cultured in Keratinocyte-SFM media. All cells were incubated in a humidified incubator at 37˚C with 5% CO_2 for 48 h prior to further experimentation.

Antibodies and reagents. The antibodies used in the present study were as follows: Rabbit anti-α-tubulin (dilution 1:2,000; cat. no. 11224-1-AP), rabbit anti-Rab7a (dilution 1:2,000; cat. no. 55469-1-AP), mouse anti-GAPDH (dilution 1:2,000; cat. no. 60004-1-Ig; all from ProteinTech Group, Inc.). The reagents used in the present study were as follows: Protein (A/G) UltraLink Resin (Thermo Fisher Scientific, Inc.), azide agarose beads (Nanocs), Thiopropyl sepharose 6B (Sigma-Aldrich; Merck KGaA), hydroxylamine (Sigma-Aldrich; Merck KGaA), N-Ethylmaleimide (NEM; Sigma-Aldrich; Merck KGaA), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich; Merck KGaA), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA; Sigma-Aldrich; Merck KGaA), R1881 (Shanghai Yuanye Bio-Technology Co., Ltd.), and 2-Brp (Sigma-Aldrich; Merck KGaA).

Protein S-palmitoylation assay in cells or supernatants. Before the S-palmitoylation assay, the cells were treated with R1881 or 2-Brp. Prior to R1881 treatment, cells were seeded with complete medium onto 10-cm-dishes ($4x10^6$ cells/dish) and incubated for 24 h. Then, the LNCaP or PC3 cells were treated with R1881 (5 nM) or DMSO in RPMI-1640 medium supplemented with 0.2% FBS. The RWPE-1 cells were treated with R1881 (5 nM) or DMSO in Keratinocyte-SFM media. After 24 h, the cells or supernatants were harvested for the S-palmitoylation assay. Prior to 2-Brp treatment, LNCaP cells were seeded with complete media onto 10-cm-dishes ($4x10^6$ cells/dish) and incubated for 40 h. The cells were then treated with 2-Brp (25 μM) or DMSO in RPMI-1640 medium supplemented with 0.2% FBS. After 4-6 h, the cells were harvested for S-palmitoylation assessment.

The S-palmitoylation assay was performed as previously described (18) with some minor modifications. In brief, the cells were homogenized in lysis buffer [10 mM sodium phosphate, 2 mM Na_2-EDTA, 0.32 M sucrose, 1% Triton X-100, 50 mM N-ethylmaleimide and Pierce protease and phosphatase inhibitor cocktail (Pierce; Thermo Fisher Scientific, Inc.); pH 7.4] for 30 min. Then, the lysates were immunoprecipitated overnight using protein A/G resin preloaded with α-tubulin antibody. The supernatants were harvested and directly incubated with 50 mM N-ethylmaleimide and Pierce protease and phosphatase inhibitor cocktail. Then, the supernatants were immunoprecipitated overnight using protein A/G resin preloaded with α-tubulin antibody.

After overnight incubation, the protein A/G resin was washed three times and incubated with elution buffer (1% SDS, 10 mM sodium phosphate, 2 mM Na_2-EDTA, 0.32 M sucrose) at 50˚C for 5 min to release α-tubulin. Eluted samples were divided into two equal portions: i) One treated with 1 M hydroxylamine and thiopropyl sepharose 6B; and ii) the other, used as the control, with 1 M Tris·HCl (pH 7.4) and thiopropyl sepharose 6B (Sigma-Aldrich; Merck KGaA). After a 2-h incubation at room temperature, sepharose beads were washed three times with washing buffer (10 mM sodium phosphate, 2 mM Na_2-EDTA, 0.32 M sucrose, 1% Triton X-100, 500 mM NaCl and 0.2% SDS). Western blots analysis was performed to determine the presence of α-tubulin.

Synthesis of palmitate probe, metabolic labeling and click chemistry. In the present study, an alkyne analogue was selected due to its physicochemical properties, similar to the wild-type fatty acid carbon chain and presented a degree of hydrophobicity that allowed a high affinity for the cell membrane (19). The alkyne group was added at the ω-position of the fatty acid. The ω-alkynyl palmitate analogue (Alk-C16) was synthesized from alcohols with internal alkynes via a zipper reaction. Subsequently, the internal alkyne was isomerized to a terminal alkyne via Jones oxidation (19).

Before the experiments, the LNCaP cells were seeded with complete media onto 6-cm-dishes ($5x10^5$ cells/dish) and incubated for 48 h. The metabolic labeling and click chemistry were performed as previously described (19) with minor modifications. The ω-alkynyl fatty acid analogue, Alk-C16, was dissolved in DMSO to generate a 50-mM stock solution and stored at -80˚C. Before cell treatment, Alk-C16 was dissolved in RPMI-1640 serum-free media supplemented with 5% BSA (fatty-acid free) at a final concentration of 100 μM. Alk-C16 was added to RPMI-1640 medium and sonicated for 15 min at room temperature. In addition, an equal volume of DMSO was added to RPMI-1640 medium as a negative control. Then, the solution containing RPMI-1640 medium and Alk-C16 or DMSO was divided into two parts: i) One was supplemented with R1881 (5 nM); and ii) the other with DMSO. The seeded LNCaP cells were washed once with PBS and treated with the four media for 24 h at 37˚C with 5% CO_2. After a 24-h incubation, the cells were washed three times and homogenized in 500 μl lysis buffer (1% Nonidet P-40, 150 mM NaCl, Pierce protease and phosphatase inhibitor cocktail and 100 mM sodium phosphate; pH 7.5) for 30 min at 4˚C. Then, the protein extracts were subjected to a probe labeling reaction for 1 h at room temperature using the following reagents: 1 mM azide agarose beads, 1 mM TCEP dissolved in water, 0.2 mM TBTA dissolved in DMSO/tert-butanol (20/80% v/v) and 1 mM $CuSO_4$ in PBS. The order of addition of the reagents to the protein extracts was critical for the reaction. Following the click-chemistry reaction, the Alk-C16-conjugated proteins were bound to the azide agarose beads. The agarose beads were washed three times with lysis buffer at room temperature. Then, the proteins bound to the azide agarose beads were digested for MS analysis.

nanoLC-MS/MS analysis. Proteomic profiling was performed on an Easy nLC1000 (Thermo Fisher Scientific, Inc.) system combined with the LTQ-Orbitrap-Elite (Thermo Fisher Scientific, Inc.) mass spectrometer as previously described (20).

Database searching. The recorded MS spectra were analyzed using MaxQuant Software (version 1.5.5.1; https://www.Maxquant.org/). The MS/MS peak list analysis was performed by searching against a forward and reverse version of the UniProtKB/Swiss-Prot human database (generated from version 2017_05; human taxonomy; 20,316 entries; http://www.uniprot.org/). The cutoff of the false discovery rate for peptide and protein identification was set to 0.01, and only peptides with ≥7 amino acidic residues were analyzed. Label-free quantitation (LFQ) was performed using the MaxQuant software on the identified razor and unique peptides in order to quantify the identified proteins (20). Gene Ontology (GO) analysis was performed using the database for annotation, visualization and integrated discovery (DAVID) software (National Institutes of Health) to assign the identified proteins with the corresponding GO terms within the category 'cellular function'.

Data processing and statistical analysis. Protein abundances normalized by the LFQ algorithm integrated in MaxQuant were log_2-transformed for further analyses. The filtering steps were performed using Microsoft Excel 2010. DanteR (version 1.0.1.1) and Perseus (version 1.5.5.3) were used to perform various types of statistical analyses including log_2 transformation, correlation plot, statistical tests and P-value adjustments (20).

Fractionation assays. Before the experiments, the cells were seeded with complete media onto 10-cm-dishes ($4x10^6$ cells/dish) and incubated for 24 h. Then, the cells were treated with R1881 (5 nM) or DMSO in RPMI-1640 medium supplemented with 0.2% FBS. After 24 h, the cells were harvested for fractionation. An Invent Biotechnologies Minute™ Plasma Membrane Protein Isolation kit (Invent Biotechnologies, Inc.) was used for total membrane fractionation according to the manufacturer's protocol. Western blot analysis was performed to analyze the protein level of α-tubulin.

Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was measured using a CCK-8 assay according to the manufacturer's protocol [MedchemExpress, (MCE)]. LNCaP cells ($5x10^3$) were seeded onto 96-well plates in RPMI-1640 medium supplemented with 0.2% FBS. After 24 h, which was selected as day 0, the cells were treated with R881 (5 nM), 2-Brp (5 μM) or DMSO. The changes in cell proliferation were analyzed for 3 consecutive days. At day 2, the cells were supplemented with 0.2% FBS. The cells were incubated with CCK-8 solution (10 μl/well) at 37˚C with 5% CO_2 for 1.5 h. The optical density value of each well was detected using a microplate reader, and the absorbance was measured at 450 nm.

Statistical analysis. Statistical analyses were performed using SPSS Statistics (version 21.0; IBM Corp.). The numerical data of each group are presented as the mean ± standard deviation. For the proteins whose palmitoylation levels was increased following androgen treatment, the significant differences in their LFO values between androgen-treated vs. untreated LNCaP cells were analyzed using a paired Student's t-test. For the cell proliferation examined by CCK-8 assay, the significant differences in cell viability between untreated vs. R1881 treated or 2-Brp treated LNCaP cells were analyzed using a paired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of androgen-induced palmitoylome using Alk-C16. In order to compare the palmitoylated protein profiles between androgen-treated and untreated LNCaP cells, Alk-C16, a palmitate fatty acid analogue, was metabolically incorporated onto the cellular proteins. Then, the Alk-C16

incorporated onto proteins was chemoselectively ligated to azide agarose beads via a Cu^{I}-catalyzed alkyne-azide [3+2] cycloaddition reaction, a type of click chemistry reaction. The Alk-C16-conjugated proteins were then ligated to the azide agarose beads. In theory, only palmitoylated proteins were conjugated to Alk-C16 and bound to the azide agarose beads. Subsequently, the proteins were digested on the agarose beads and subjected to MS and to an informatics-assisted label-free quantitation (Fig. 1).

To identify the proteins exhibiting increased levels of palmitoylation following androgen treatment, three groups of androgen-treated and untreated LNCaP cells were investigated. In total, 927 proteins exhibiting a Mascot score >2 were identified ($P<0.05$). The outlier proteins and contaminant proteins were removed. The 927 proteins were subjected to LFQ using MaxQuant software. In these 927 proteins, the LFQ values were positively correlated with the palmitoylation levels. Among these 927 proteins, 504 proteins were identified to be palmitoylated preliminarily, and their LFQ values were >0 in ≥2 replicates of untreated LNCaP cells. Among these 504 proteins, there were a number of well-known palmitoylated proteins, including α- and β-tubulin, fatty acid synthase, catenin Δ1 and ubiquinol-cytochrome *c* reductase core protein 1 (21,22). Among the 504 proteins, 96 candidates exhibited palmitoylation levels that were increased following androgen treatment, and their LFQ values were significantly upregulated (fold change >1.5; $P<0.05$) in ≥2 samples of androgen-treated vs. untreated LNCaP cells (Table SI). GO analysis revealed that the functions of the 96 proteins were categorized as: 25% 'metabolism', 12% 'protein folding', 8% 'cytoskeleton', 7% 'protein *de novo* biosynthesis', 4% 'protein modification' and 2% 'vesicle trafficking', among others (Fig. 2). The proteins involved in 'metabolism' were included in the following categories: 7% 'glycometabolism', 7% 'respiratory electron transport-chain', 6% 'lipid metabolism', 2% amino acid metabolism' and 1% 'tricarboxylic acid cycle', among others.

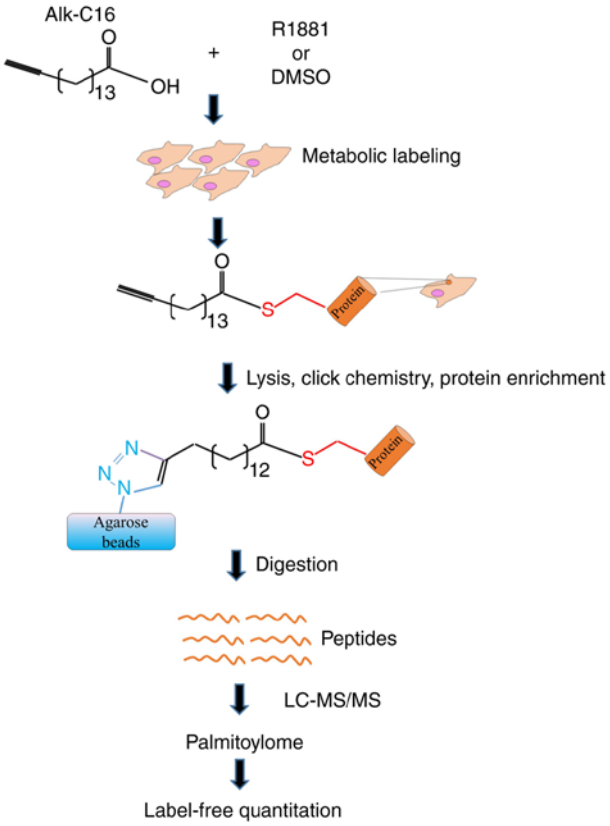

Figure 1. Schematic overview of the approach used for the identification of the androgen-induced palmitoylome using click chemistry. Cultured cells were incubated with Alk-C16, a click palmitate probe. Treated cells were divided into two groups: One was treated with R1881, an androgen, and the other was treated with DMSO, used as the control. The cells were harvested and lysated. The Alk-C16-conjugated proteins were bound to the azide agarose beads by click chemistry. The proteins bound to the agarose beads were digested with trypsin. The supernatant was subjected to mass spectrometry analysis in order to investigate the palmitoylome using label-free quantification.

Androgen treatment promotes the palmitoylation level of α-tubulin. Among the 96 proteins exhibiting increased palmitoylation following androgen treatment, 7% were cytoskeleton-related proteins including tubulin α 4A (TUBA4A), TUBA1B, tubulin β 2C, tubulin β chain, myosin heavy chain 9, profilin-1, actinin α 4 and filamin-B (Fig. 3A-H). Notably, the palmitoylation levels of both α-tubulin and β-tubulin were significantly (>1.5-fold) higher in androgen-treated LNCaP cells compared with untreated LNCaP cells (Fig. 3A-D). The protein with the highest LFQ value was TUBA1B in both androgen-treated and untreated LNCaP cells.

α-tubulin is a well-known S-palmitoylated protein (22). To further investigate the role of androgen treatment on α-tubulin palmitoylation in LNCaP cells, a thiopropyl protein captivation assay was performed. As revealed in Fig. 4A, α-tubulin proteins were observed to be associated with the thiopropyl beads following treatment with hydroxylamine, but not following treatment with Tris·HCl, used as the control. Following treatment with R1881, an androgen, the palmitoylation level of α-tubulin was significantly upregulated. The present results were consistent with the aforementioned MS results. Collectively, the present results indicated that androgen promoted the palmitoylation level of α-tubulin in LNCaP cells.

In addition to the results observed in LNCaP cells, thiopropyl captivation assay of S-palmitoylated proteins was performed in other cell types in order to investigate whether androgen promoted the levels of tubulin palmitoylation in RWPE-1 cells, normal prostate epithelial cells and in PC3 cells, which exhibit a decreased expression level of AR (23). As revealed in Fig. 4B and C, there were no significant differences in the palmitoylation levels of α-tubulin between RWPE-1 or PC3 cells treated with R1881 or DMSO.

Since androgen significantly increased the palmitoylation level of α-tubulin, the effect of androgen on the function of α-tubulin was investigated. Considering that palmitoylation is essential for membrane association, plasma membrane fractions were separated from androgen-treated and untreated cells. The protein expression levels of plasma membrane-associated α-tubulin were compared between the two samples. As revealed in Fig. 4D, the protein expression level of plasma membrane-associated α-tubulin in androgen-treated cells was higher compared with untreated cells. The present result indicated that androgen treatment enhanced the plasma membrane association of α-tubulin, in line with the aforementioned

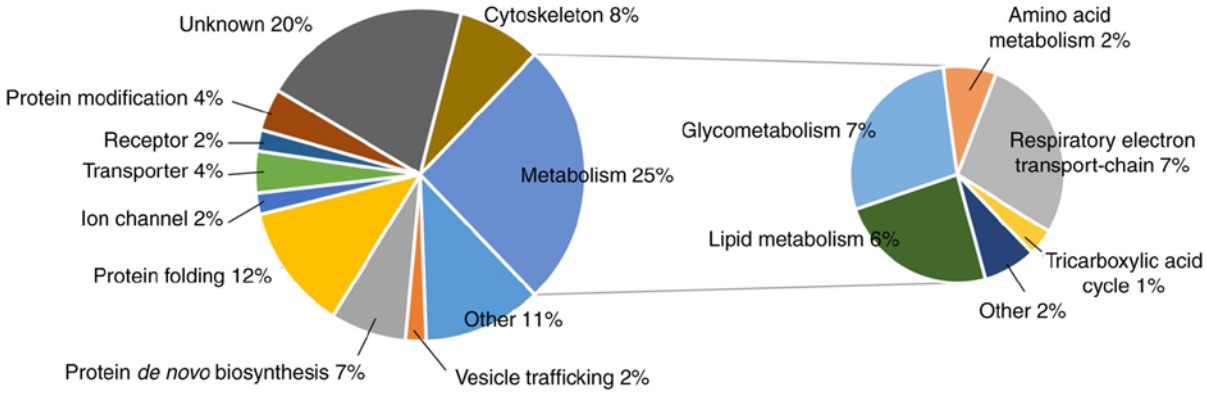

Figure 2. Pie diagram illustrating the function of the protein exhibiting increased palmitoylation levels following androgen treatment.

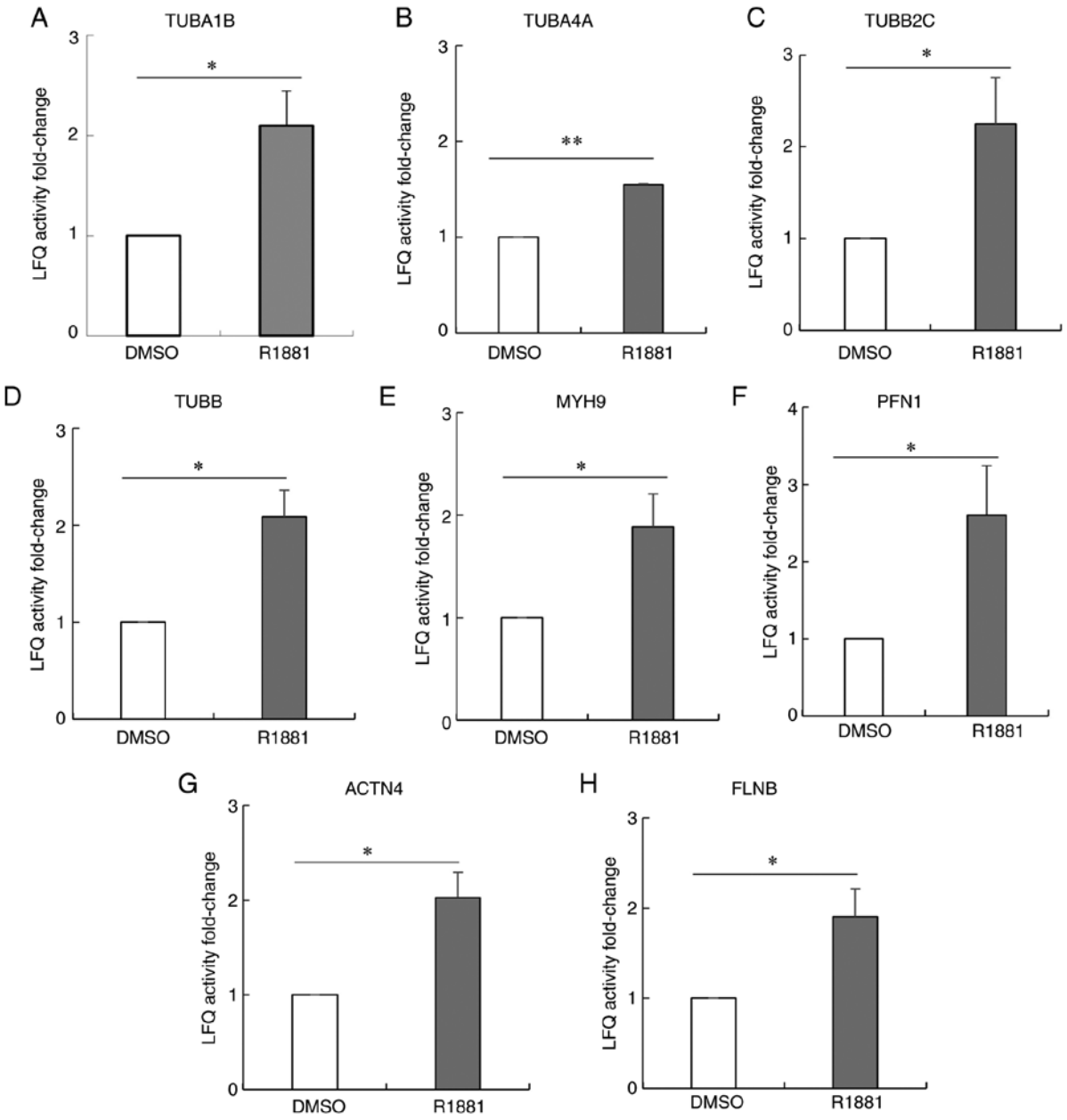

Figure 3. Androgen-enhanced palmitoylation of cytoskeleton-related proteins in LNCaP cells identified following palmitoylome analysis. The palmitoylation levels of cytoskeleton-related proteins (A-H) were significantly (>1.5-fold) higher in androgen-treated LNCaP cells compared with untreated cells. (A) TUBA1B, (B) TUBA4A, (C) TUBB2C, (D) TUBB, (E) MYH9, (F) PFN1, (G) ACTN4, (H) FLNB. Data are presented as the mean ± SD from three independent experiments. Data were analyzed using a paired Student's t-test. *P<0.05, **P<0.01. TUBA1B, tubulin α 1b; TUBA4A, tubulin α 4a; TUBB2C, tubulin β 4B class IVb; TUBB, tubulin β class I; MYH9, myosin heavy chain 9; PFN1, profilin-1; ACTN4, actinin α 4; FLNB, filamin B.

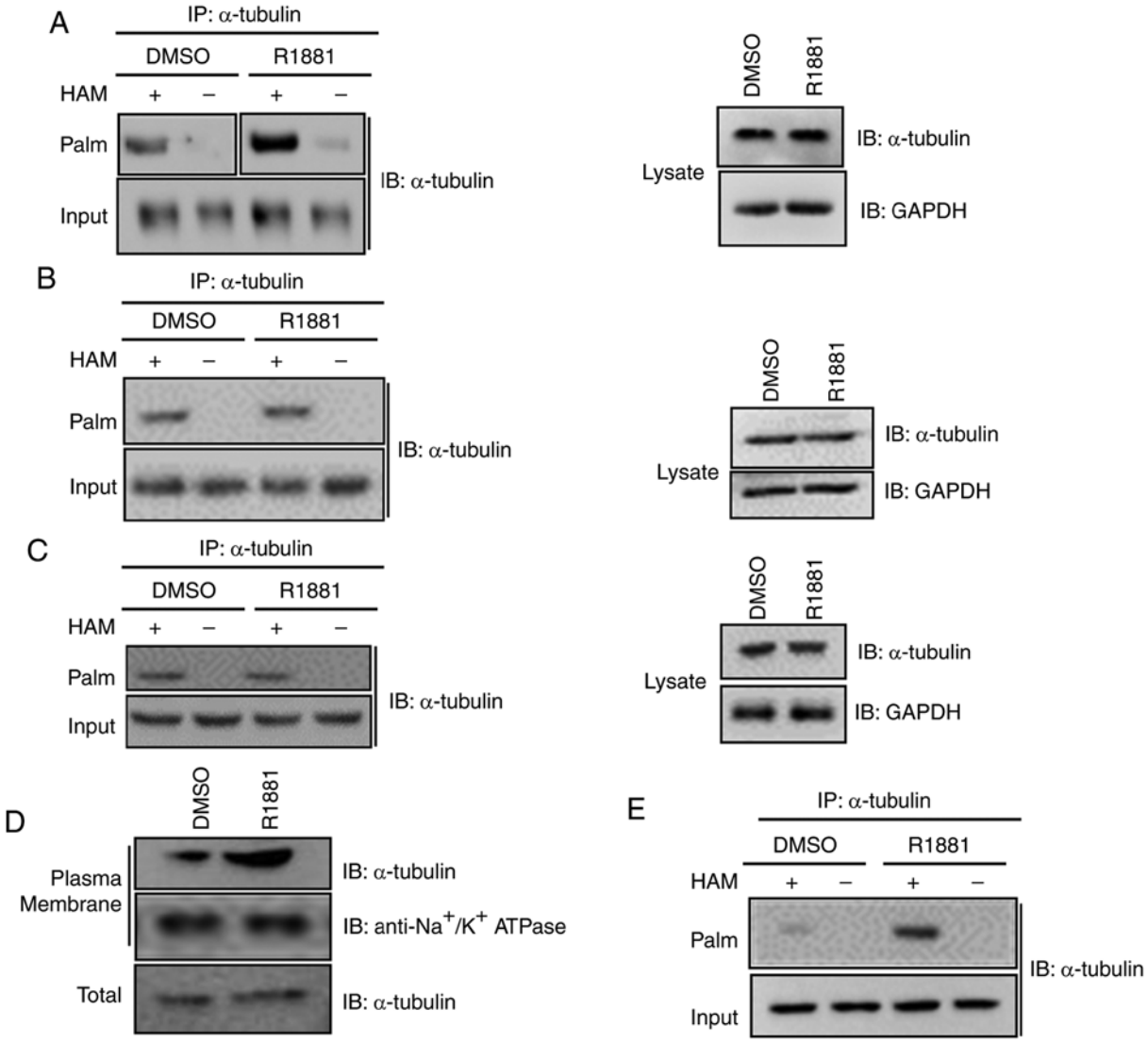

Figure 4. Androgen treatment promotes the palmitoylation level of α-tubulin in LNCaP cells. (A) LNCaP cells, (B) RWPE-1 cells and (C) PC3 cells were treated with R1881 or DMSO, and a thiopropyl captivation of S-palmitoylated protein assay was performed to assess the palmitoylation level of α-tubulin. (D) Androgen treatment enhanced the plasma membrane association of α-tubulin. Plasma membrane fractions were separated from androgen-treated and untreated cells. The protein expression levels of the plasma membrane-associated α-tubulin were compared between the two samples. (E) The supernatants of LNCaP cells treated with R1881 or DMSO were analyzed by thiopropyl captivation of S-palmitoylated protein assay to assess the palmitoylation level of α-tubulin. HAM, hydroxylamine; Palm, palmitoylation; IB, immunoblot; IP, immunoprecipitation.

results indicating that androgen treatment increased the palmitoylation levels of α-tubulin (Fig. 4A).

In addition, thiopropyl captivation of S-palmitoylated proteins was performed to examine whether androgen promoted the level of α-tubulin palmitoylation in the supernatants of LNCaP cells. As revealed in Fig. 4E, the palmitoylation level of α-tubulin was significantly higher in R1881-treated cells compared with untreated cells, in line with the aforementioned results.

In summary, androgen treatment promoted the palmitoylation level of α-tubulin in LNCaP cells, an AR-dependent prostate cancer deprived-cell line.

Androgen treatment promotes the palmitoylation level of Ras-related protein Rab-7a (Rab7a). Among the 96 proteins exhibiting increased palmitoylation levels following androgen treatment, there were two members of the Rab protein family, Rab7a and Rab5c (Fig. 5A and B). Their LFQ values were lower compared with α-tubulin. In order to further examine whether androgen promoted the palmitoylation level of the two members of Rab family in LNCaP cells, thiopropyl captivation of S-palmitoylated proteins was performed. As revealed in Fig. 5C, Rab7a proteins were associated with thiopropyl beads following treatment with hydroxylamine, but not following treatment with Tris·HCl, used as the control. Following treatment with the androgen R1881, the palmitoylation level of Rab7a was significantly upregulated. The present results were consistent with the aforementioned MS results (Fig. 5A). Collectively, the present results indicated that androgen treatment promoted the palmitoylation level of Rab7a in LNCaP cells.

The proliferation of LNCaP cells is promoted by androgen treatment and inhibited by palmitoylation inhibitor. To investigate the effect of androgen-induced palmitoylation of

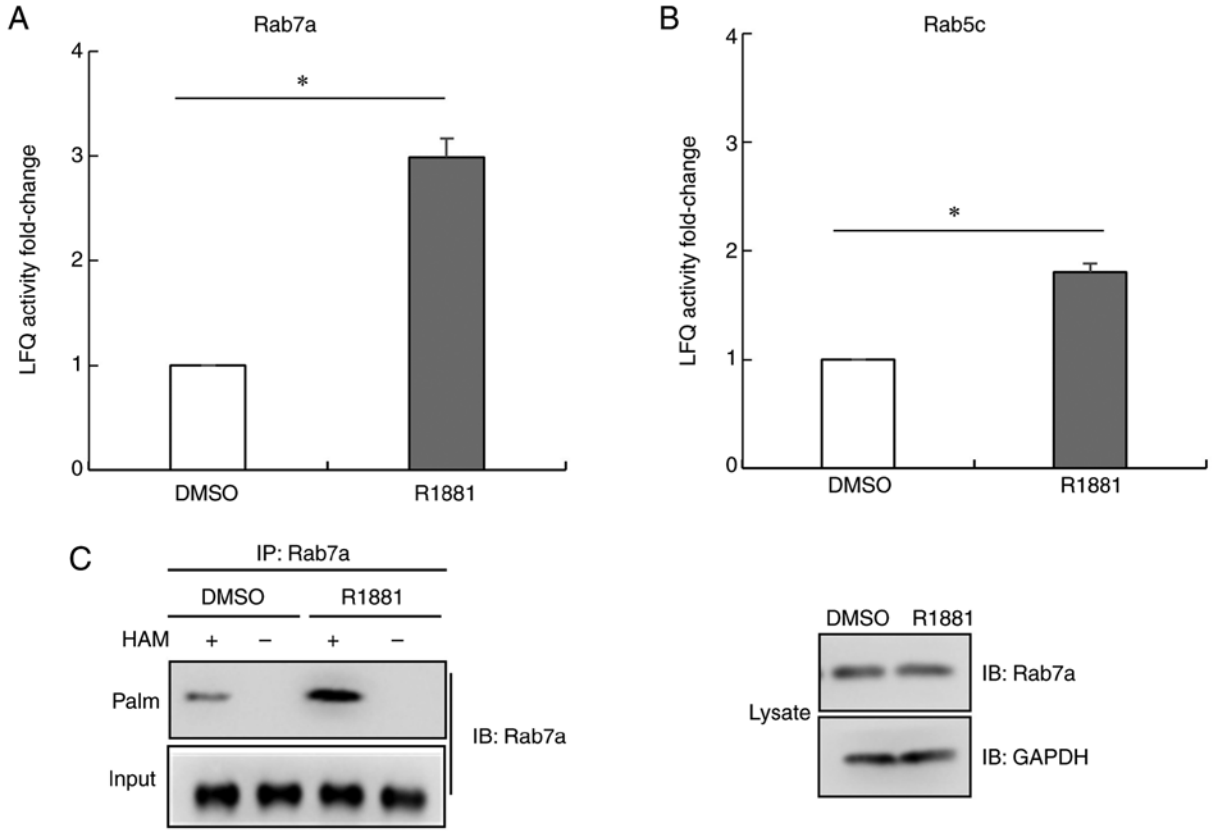

Figure 5. Androgen treatment promotes the palmitoylation levels of Rab family members in LNCaP cells. The palmitoylation levels of Rab family members (A and B) were significantly (>1.5-fold) higher in androgen-treated LNCaP cells compared with untreated cells. (A) Rab7a and (B) Rab5c. (C) LNCaP cells were treated with R1881 or DMSO, and a thiopropyl captivation of S-palmitoylated protein assay was performed to assess the palmitoylation level of Rab7a. Data in (A) and (B) are presented as the mean ± SD from three independent experiments. The data were analyzed using a paired Student's t-test. *P<0.05. HAM, hydroxylamine; Palm, palmitoylation; IB, immunoblot; IP, immunoprecipitation.

α-tubulin and Rab7a on the proliferation of LNCaP cells, a CCK-8 assay was performed. As revealed in Fig. 6A, R1881 treatment induced the proliferation of LNCaP cells. By contrast, following treatment with 2-Brp, a palmitoylation inhibitor, cell proliferation was inhibited (Fig. 6B). S-palmitoylated assay results indicated that 2-Brp treatment reduced the palmitoylation levels of α-tubulin and Rab7a compared with untreated cells (Fig. 6C and D). The present results indicated that palmitoylation of α-tubulin and Rab7a were critical for LNCaP cell proliferation.

Discussion

The clickable palmitate probe Alk-C16 can be used to identify novel palmitoylated proteins, examine the subcellular distribution of palmitoylated proteins and investigate fatty acid substrates. The use of Alk-C16 presents certain advantages, including the potential to be used in cultured cells to detect proteins that have been palmitoylated during metabolic labeling in different conditions, such as gene overexpression, gene knockdown and drug treatment (16,17). Notably, Alk-C16 can be combined with quantitative proteomics to quantify the levels of palmitoylated proteins.

The present study aimed to identify the androgen-induced palmitoylated proteins by comparing the palmitoylome profile of androgen-treated LNCaP cells with untreated cells. The present results indicated that androgen promoted the palmitoylation of α-tubulin in LNCaP cells (Figs. 3A, B and 4A). In previous studies, α-tubulin was revealed to be palmitoylated, and this modification is essential to anchor microtubule filaments to the plasma membrane, influencing cellular transport and cell division (24-26). In addition, in the present study, androgen treatment was revealed to increase the palmitoylation level of Rab7a, a member of the Rab family (Fig. 5A and C). Rab7a localizes in late endosomes and regulates the trafficking from early endosomes to late endosomes and from late endosomes to lysosomes (27,28). Rab7a is essential for lysosomal biogenesis, localization and function, and lysosomes are involved in vesicular trafficking and in the degradation of signaling receptors (29). Rab7a palmitoylation is required for the spatiotemporal recruitment of the retromer complex and for an efficient endosome to *trans*-Golgi network trafficking of the lysosomal sorting receptor; however, Rab7a palmitoylation is not involved in the membrane anchoring (30). Rab7a controls vesicle trafficking by cooperating with α-tubulin (31). Therefore, the palmitoylation of α-tubulin and Rab7a is critical for the vesicle trafficking from the plasma membrane to the lysosomes, which are involved in the degradation of some signaling receptors. Functional analysis indicated that androgen promoted the palmitoylation of α-tubulin and Rab7a in LNCaP cells, inducing cell proliferation (Fig. 6A). By contrast, 2-Brp, which reduced the palmitoylation of α-tubulin and Rab7a (Fig. 6C and D), inhibited cell proliferation (Fig. 6B).

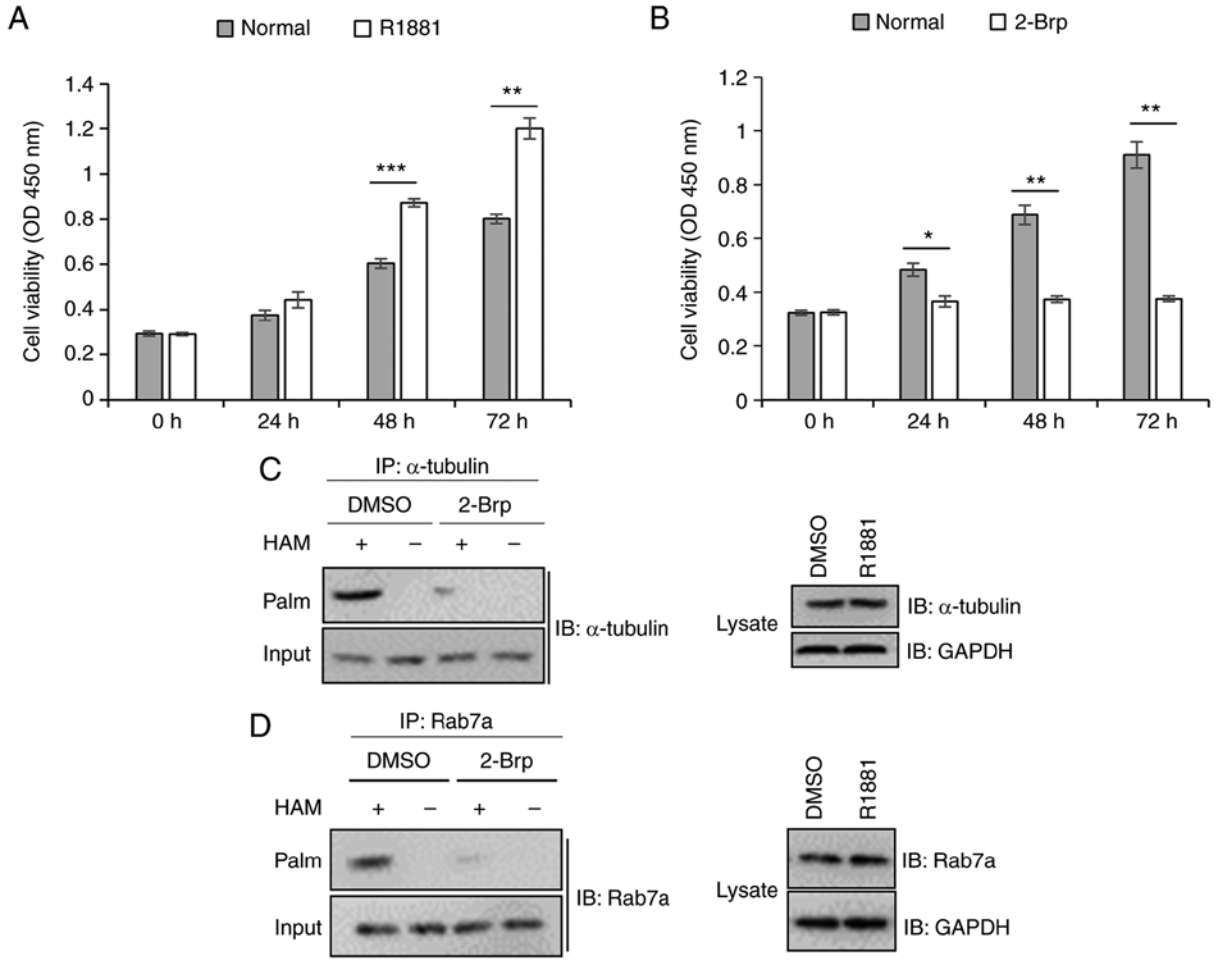

Figure 6. Proliferation of LNCaP cells is increased following androgen treatment and inhibited following 2-Brp treatment. LNCaP cells were treated with (A) R1881 or(B) 2-Brp, and cell proliferation was investigated using CCK-8 assays. LNCaP cells were treated with 2-Brp or DMSO, and a thiopropyl captivation of S-palmitoylated protein assay was performed to assess the palmitoylation level of (C) α-tubulin and (D) Rab7a. Data in (A and B) are presented as the mean ± SD from three independent experiments. The data were analyzed using a paired Student's t-test. *P<0.05, **P<0.01, ***P<0.001.

Collectively, the present results indicated that androgen-induced palmitoylation of α-tubulin and Rab7a promoted cell proliferation by degrading inhibitors of cell proliferation. Therefore, palmitoylation of α-tubulin and Rab7a may represent novel potential therapeutic targets for treating prostate cancer. Notably, in the supernatants of LNCaP cells, the palmitoylation level of α-tubulin was significantly higher in the R1881-treated cells compared with untreated cells (Fig. 4E). Therefore, further studies are required to identify whether the palmitoylation level of α-tubulin in the serum of patients with prostate cancer may represent a potential novel biomarker for early-stage prostate cancer.

The present results indicated that 25% of the proteins exhibiting an increase in palmitoylation following androgen treatment were involved in metabolism. However, it is challenging to examine the regulatory mechanism underlying the palmitoylation of all these proteins, which are involved in various metabolic processes. In LNCaP cells, androgen treatment, which promoted the palmitoylation of α-tubulin and Rab7a, induced cell proliferation, whereas 2-Brp, which reduced the palmitoylation of α-tubulin and Rab7a, inhibited cell proliferation. Palmitoylation of α-tubulin and Rab7a were identified to be involved in cell proliferation and may represent new targets for developing novel treatments against prostate cancer. In addition, a high level of α-tubulin palmitoylation may represent a novel biomarker for early-stage prostate cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WL and GZ designed the study. WL, JZ, LZ, JC, FS and JJ performed experiments. WL, FX, ML and GZ analyzed the data. WL, FX, ML and GZ wrote the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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