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Original Article

Molecular mechanism of prostate cancer cell apoptosis induced by busulfan via adjustment of androgen receptor phosphatization

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Abstract: Objective: To probe killing effect of busulfan to prostate cancer cell without androgen and the influence of androgen receptor phosphatization and analyze its molecular mechanism. Methods: prostate cancer cell line 22RV1, LAPC4 and LNCaP treated with busulfan under androgen-free condition underwent CCK-8 examination to probe killing ability of the medicine. Flow cytometry was used to check the influence of busulfan on apoptosis rate of prostate cancer cell line LAPC4. Expression level of androgen receptor (AR), Src and Ack1 and change in phosphatization of AR after busulfan treatment were measured by RT-PCR and Western blotting. Finally, influence o proliferation ability and apoptosis of LAPC4 were measured using EGF-busulfan co-processing. Results: Significant dose-dependency was observed as killing ability rises with higher busulfan concentration ($p<0.05$). Significant improvement in prostate cancer cell inhibition ability of busulfan was also observed with prolonging of time ($p<0.05$). Then we discovered, as indicated by flow cytometry, that busulfan inhibits prostate cancer cell LAPC4 proliferation by strengthening its apoptosis ($p<0.05$), which showed significant dose- and time-dependency. Detection of AR expression and phosphatization level showed no significant influence on mRNA and protein expression level of AR made by busulfan. However, decline of phosphatization level at AR Y534 site was positively related to busulfan treatment time. Busulfan was found to be inhibitory to Src kinase induced by EGF and level of resulting AR phosphatization in our further probe into the mechanism of busulfan influence on phosphatization level at AR Y534 site. Nude mice experiment indicated that busulfan was inhibitory to protein expression of AR downstream target gene prostate specific antigen (PSA) and human tissue kallikrein2 (hk-2), thus inhibited in vivo tumorigenic ability of prostate cancer cells. Conclusion: Busulfan was significantly inhibitory to prostate cancer cell proliferation by inhibiting phosphatization of Src kinase at AR Y534 site.

Keywords: Busulfan, androgen receptor, tyrosine kinase, phosphatization, Src kinase

Introduction

Androgen and its receptor have significant contribution in the occurrence and development of prostate cancer. After ligand binding, androgen receptor (AR) may act as nuclear factor in activation of transcription of several proteins closely relevant to cell proliferation and apoptosis, thus sustains the growth of tumor cells [1, 2]. Rejection of androgen by castration or medicine is an effective treatment to prostate cancer, but its efficacy is temporary, as the disease develops into castration-resistant prostate cancer (CRPC) [3, 4]. In early stage of ADPC (androgen-dependent prostate cancer) androgen deprivation treatment, tumor growth and PSA expression in AR downstream gene are inhibited due to inhibition of AR transcription activity caused by loss of ligand. AR transcription activity recovers and then strengthens with the prolonging of treatment (ranging from 3-6 months to 2 years), resulting in faster tumor growth and higher PSA expression, marking the formation of AIPC [5-7]. This phenomenon indicates the key role of AR activation under low testosterone condition in occurrence of CPRC [8]. However, mechanism of activation of the androgen receptor under such circumstance is still unknown.

AR over-expression was facilitatory to generation of prostate xenograft while AR knockout

inhibits its tumor origin, as was reported by Chen et al. [9]. Gene amplification, point mutation, over-expression of AR or co-activation factors and androgen produced by tumor itself were among possible mechanisms of AR activation. Recent studies discovered that AR phosphatization caused by androgen-independent intracellular signal transduction is an important way of AR activation [10-12]. EGFR (epidermal growth factor receptor) enhances transcriptional activity of AR by interacting with TIF2 (transcriptional intermediary factor 2). HER2 (human epidermal receptor 2) activates transcriptional function of AR by enhancing its protein stability and DNA binding force [13]. Studies also suggest that EGF (epidermal growth factor) may enhance AR activity in malignant prostate cancer [14].

Busulfan (Bu), or myleran is a nitrogen mustard with the chemical name 1, 4-Dimethanesulfonoxybutane. It's a representative methyl sulfonate medicine. As a cell cycle nonspecific agent, the drug damages form and function of DNA in a cell via alkylation with its guanine in GI/GO phase [15, 16]. Since 1952, busulfan has been used in treatment of CML (chroniomyelocyti leukemia). It's a traditional chemotherapeutic drug for CML that showed exact effect in most chronic patients [17]. It's also been widely used in preparation of male rat sterility model in recent years [18, 19]. Common medicine as it is, its molecular mechanism is still unknown. The study aimed at probing influences of busulfan on prostate cancer cell proliferation and androgen receptor phosphatization and the mechanism behind them.

Materials and methods

Main reagents

Busulfan was from Sigma. Annexin-V and PI were from Becton Dickinson. RPMI-1640 (with/without phenol red) was from GIBCO. Common fetal bovine serum and fetal bovine serum after active carbon filtration were from Hycolone. Androgen receptor (AR) Tyr-534 phosphatization specific antibody was from Abcam. Monoclonal antibody against AR was from Milipore. Src antibody was from Abcam. PSA and hk-2 antibody were from Santa Cruz. RNA isolating reagent was from Invitrogen. RIPA was self-made. Reverse transcription kit AMV RT and PCR kit Hot-start PCR kit were from Promega. CCK-8 cell proliferation assays kit was from Keygentec, a Nanjing manufacturer.

Cell culture and medicine treatment

Prostate cancer cell line 22RV1, LAPC4 and LNCaP were from ATCC. Mother LNCaP cells were cultured in 10% (v/v) fetal bovine serum/1640 culture medium with phenol red. LNCaP cells of 80% cell density were washed 3 times in PBS, then added to 10% fetal bovine serum/1640 culture medium with phenol red to establish androgen-independent LAPC4 cell line.

Establishing adenovirus vector carrying PKA gene and hk2 gene

Open reading frame of PSA and hk2 was amplified by PCR according to their sequence in Gene Bank. PSA and hk2 open reading frames were cloned into adenovirus vector to establish adenovirus vector Ad-PSA and Ad-hk2. Then viruses were packaged.

Establishing Wistar rat model bearing prostate tumor

Wistar rats were purchased by the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and fed to 4-8 weeks old in laboratory of SPF class. Process of animal experiment was under corresponding agreements. Prostate cancer LAPC4 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum under 5% CO_2, 37°C temperature and saturated humidity. LAPC4 cells were digested by 0.25% trypsin at beginning of their confluence before being centrifuged for 5 min at 1000 rpm. Supernatant was discarded. Single cell suspension of LAPC4 was prepared at concentration of 1.5×10^7 cells/ml. Subcutaneous injection of 3×10^6 LAPC4 cells floating on 50 μL PBS into Wistar rats was performed.

Detecting toxicity of drugs to prostate cancer cells using CCK-8 method

Influence of busulfan on proliferation ability of prostate cancer cells and its toxicity to prostate cancer cells were detected using CCK-8 detection kit. Prostate cancer cell line 22RV1, LAPC4 and LNCaP was divided into control group and busulfan group respectively. Medicine concentration gradient of busulfan was set (0 mg/L,

5 mg/L, 20 mg/L, 50 mg/L, 100 mg/L, 200 mg/L). Main steps: Single cell suspension of cells at logarithmic growth phase was prepared and inoculated to a 96-well-plate (1×10^3 cells/cell) for medicine inoculation. 6 parallel holes were set for each concentration. After 72 h of treatment, samples were moved to incubator under 5% CO_2, 37°C temperature and saturated humidity for 1.5 h before OD measurement using enzyme mark instrument (with 450 nm light) Steps were repeated 3 times. Samples of suitable concentration (60 mg/l) were selected to undergo time gradient test: CCK-8 solution was added to culture medium every 24 h since the second day to detect 450 nm light concentration using enzyme mark instrument after 1.5 h of incubation. The test was repeated 3 times. Inhibition rate was calculated as follows: Inhibition rate (%) = (control group-treatment group)/(control group-blank group) ×100%. IC50 was calculated using median-effect formula.

Expression level was measured employing real-time quantitative PCR

RNA of 5×10^6 cells, whose purity was to be tested with NanoDrop-1000, were extracted using chloroform after Trizol lysis. cDNA synthesized with reverse transcription kit was used as template for RNA reverse transcription employing random primers. mRNA expression level of AR, PSA and hk2 was measured with that of GAPDH as internal parameter using real-time quantitative PCR kit. Sequence of primers and probes was as follows: PSA mRNA upstream, 5'-GGCAGCATTGAACCAGAGGAG-3'; PSA downstream, 5'-GCATGAACTTGGTCACCTTCTG-3'; hk2 upstream, 5'-GCCTTAGACCAGATGAAGACTCCA-3'; hk2 downstream, 5'-GCCCAGGACCTTCACAACATC-3'; GAPDH upstream, 5'-GTCATGGGTGTGAACCATGAGA-3'; GAPDH downstream, 5'-GGTCATGAGTCCTTCCACGATAC-3.

Expression of protein was measured using Western blotting

Ultrasonic lysis of cells was performed for 10 min after put on ice for 30 min. 15 min of 12000 rpm centrifuge of lysate was performed at 4°C. Concentration of protein was measured using protein assay kit. Protein samples were collected after 10 min of heat bath at 95-100°C with 5× Loading buffer and added to 10% SDS-PAGE to perform electrophoretic separation. Samples were sealed with 5% milk after 2 h of 200 mA gel transmembrane. Then AR (1:1000), P-AR-Tyr534 (1:500), mouse anti-Src (1:500), mouse anti-PSA (1:500), mouse anti-hk-2 (1:500) and GAPDH (1:1000) primary antibody were added to the samples and underwent three times of 2 h room-temperature incubation and 15 min TBS membrane cleaning before secondary antibody marked with HPR was added and underwent 1 h room-temperature incubation, 15 min TBS cleaning for ECT exposure and band analysis.

Apoptosis rate was measured by flow cytometry

LNCaP cells were cultured in PRMI-1640 medium with 10% PBS to the concentration of 1×10^6 cells/ml and digested by trypsin before being implanted to a 6-well plate. They were divided later into control group, busulfan (30 mg/L) group, busulfan (60 mg/L) group and busulfan (100 mg/L) group as they reached 70% of the container. Single LNCaP cells were obtained by separating cells digested by trypsin after 12 h busulfan (of concentrations mentioned above) pretreatment. 1×10^6 cells were collected and double stained by Annexin-V marked with luciferin APC and PI (Becton Dickinson). Finally, apoptosis rate was measured using flow cytometry. All tests were repeated for 3 times.

Statistical analysis

SPSS18.0 was used for data analysis. Measurement data were presented as x±s and received t test, numeration data were presented as ratio and received X^2 test, $P<0.05$ was considered as statistically significant.

Results

Busulfan effectively inhibited survival of prostate cancer cells

While literal materials reporting the adoptability of busulfan treatment on CML were accessible, we found no report of busulfan influence on prostate cancer cells. Thus we employed CCK-8 test to measure survival rate of prostate cancer cell line 22RV1, LAPC4 and LNCaP at 48 h after treating with busulfan of varied concentrations (0 mg/L, 5 mg/L, 20 mg/L, 50 mg/L, 100 mg/L and 200 mg/L) in order to probe busulfan influence on survival rate of

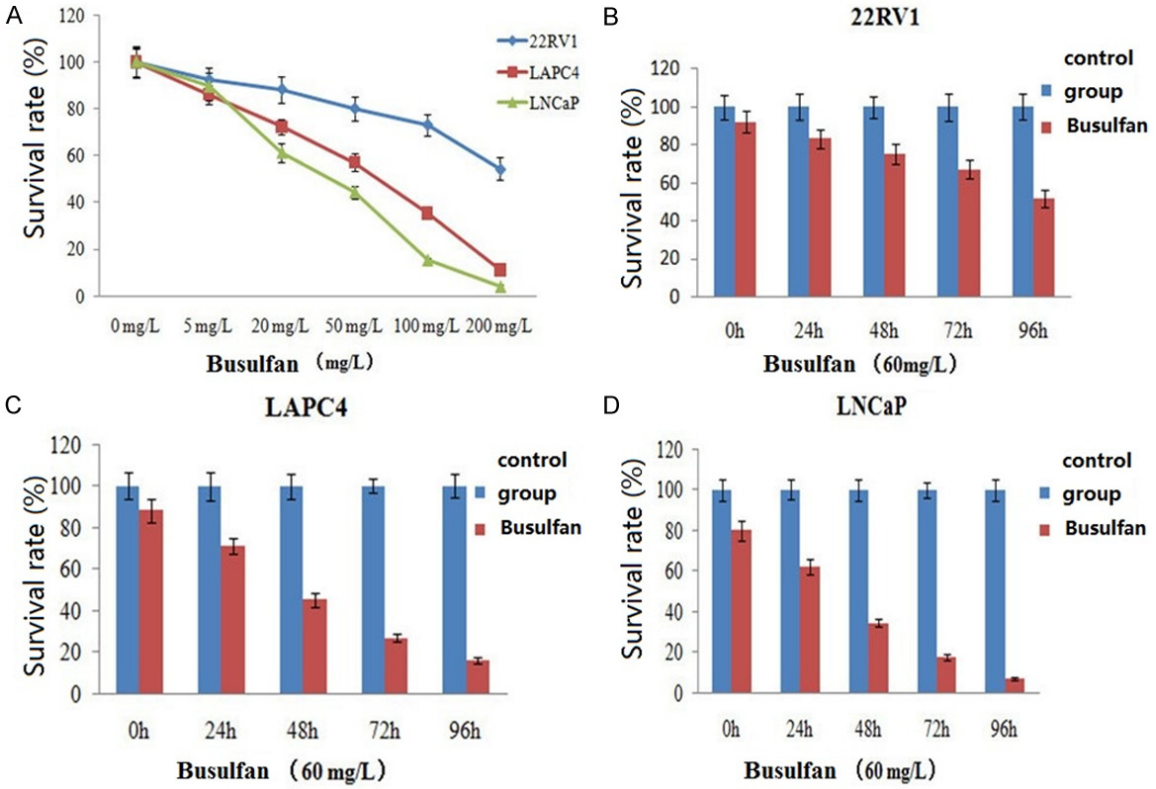

Figure 1. Busulfan effectively inhibited survival of prostate cancer cells. A. CCK-8 test probing influence of varied busulfan dose (0 mg/L, 5 mg/L, 20 mg/L, 50 mg/L and 100 mg/L) on prostate cancer cell 22RV1, LAPC4 and LNCaP; B. CCK-8 test probing 60 mg/L busulfan influence under time gradient (0 h, 24 h, 48 h, 72 h and 96 h) on survival ability of prostate cancer cell 22RV1; C. CCK-8 test probing 60 mg/L busulfan influence under time gradient on survival ability of prostate cancer cell LAPC4; D. CCK-8 test probing 60 mg/L busulfan influence under time gradient on survival ability of prostate cancer cell LNCaP.

prostate cancer cells. Results indicated obvious dose-dependency as killing ability rises with higher busulfan concentration. Survival rate, especially that of LAPC4 and LNCaP dropped significantly with busulfan concentration ≥50 mg/L ($p<0.05$, $p<0.05$), as shown in **Figure 1A**. Then we measured survival rate of prostate cancer cell line 22RV1, LAPC4 and LNCaP at 0 h, 24 h, 48 h, 72 h and 96 h after being treated with 60 mg/l busulfan and noticed time-dependency of the killing effect on prostate cancer cells, especially LAPC4 and LNCaP cells of busulfan ($p<0.05$, $p<0.05$) (**Figure 1B-D**).

Busulfan inhibited survival of prostate cancer cells by inducing cell apoptosis

Results of the study suggested that busulfan hold obvious time-dependent and dose -dependent influence on prostate cancer cell survival, though its molecular mechanism was still unknown. We employed flow cytometry to measure influence of busulfan of different concentrations (0 mg/L, 30 mg/L, 60 mg/L and 100 mg/L) on apoptosis proportion of prostate cancer cell LAPC4 since cell apoptosis is the main mechanism of decline in survival rate. Results showed that cell apoptosis rate raised significantly with higher busulfan concentration, suggesting obvious dose-dependency. Apoptosis rate of prostate cancer cells went beyond 50% when busulfan concentration ≥60 mg/L. Apoptosis rate was 58.75% at 60 mg/L, and 75.57 for 100 mg/L, showing very significant difference as compared to control group ($p<0.01$, $p<0.01$) (**Figure 2A**). Then we probed into time-dependency of busulfan induction of cell apoptosis. We treated 2 groups of prostate cancer cells with 60 mg/L busulfan for 12 h and 24 h respectively, and found that 2 groups showed apoptosis rate of 18.95% and 41.15% respectively, suggesting significant time-dependency.

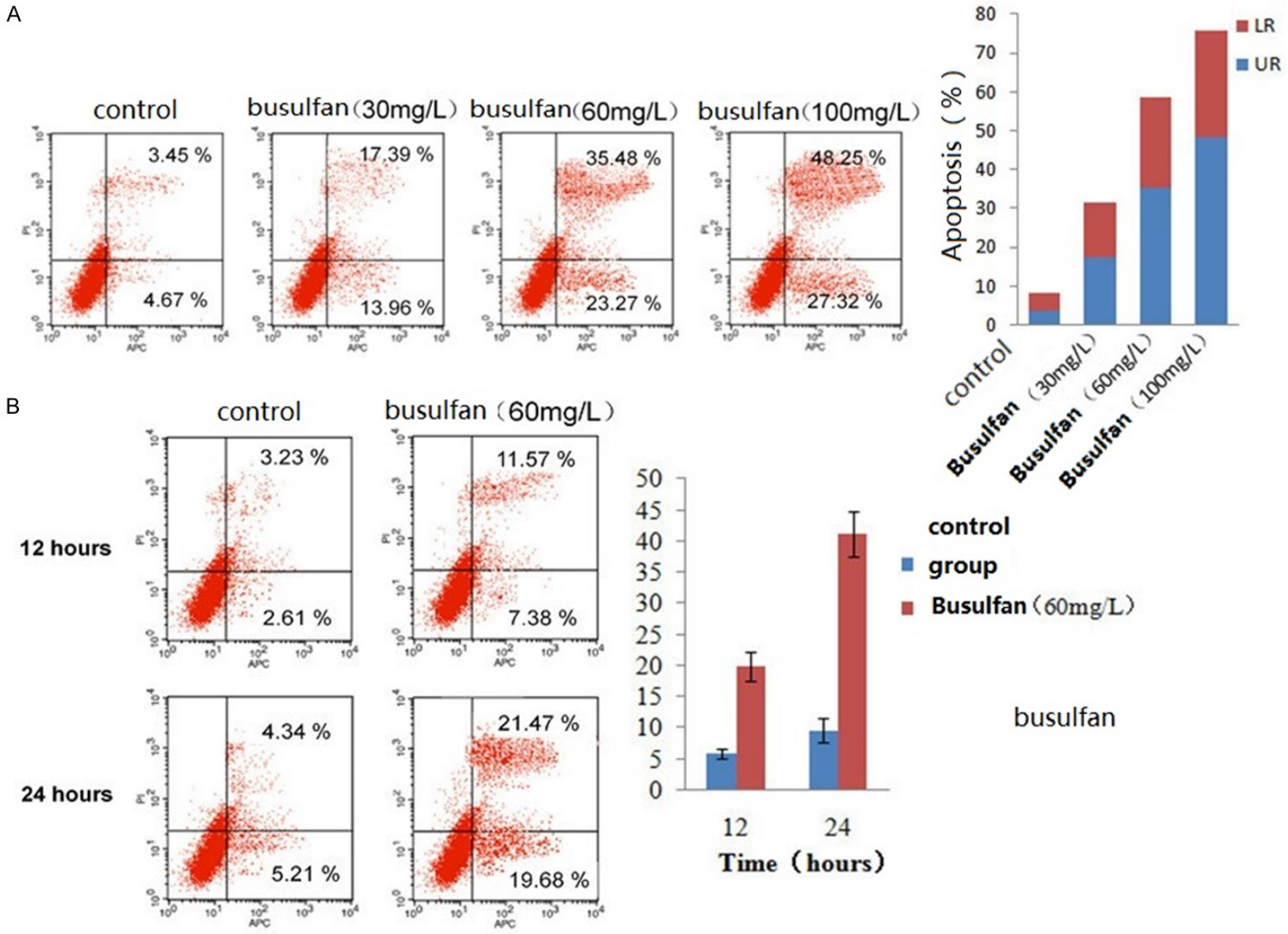

Figure 2. Busulfan effectively inhibited survival of prostate cancer cells by inducing cell apoptosis. A. Flow cytometry probing influence of varied busulfan dose (0 mg/L, 30 mg/L, 60 mg/L and 100 mg/L) on apoptosis of prostate cancer cell line LAPC4 at 24 h; B. Flow cytometry probing influence of time gradient (12 h and 24 h) on apoptosis of prostate cancer cell line LAPC4 treated with 60 mg/L busulfan.

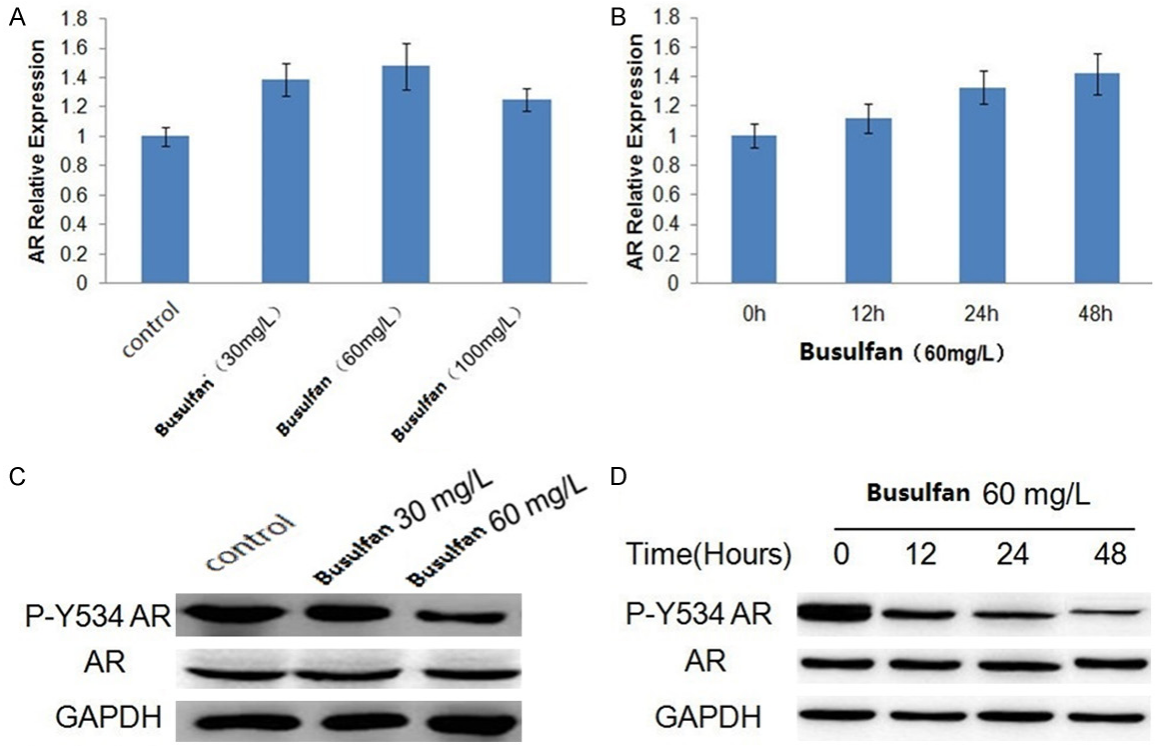

Figure 3. Busulfan lowered phosphatization level of prostate cancer cells significantly. A. RT-PCR probing influence of varied concentration (0 mg/L, 30 mg/L, 60 mg/L and 100 mg/L) of busulfan on expression level of AR mRNA; B. RT-PCR probing influence of varied length (0 h, 12 h, 24 h and 48 h) of busulfan treatment on AR mRNA expression; C. Western blotting probing influence of varied concentration (0 mg/L, 30 mg/L and 60 mg/L) of busulfan at 24 h after treatment on AR protein expression level and AR phosphatization at Y534 site; D. Western blotting probing influence of varied length (0 h, 12 h, 24 h and 48 h) of 60 mg/L busulfan treatment on AR protein expression level and AR phosphatization at Y534 site.

Busulfan treatment may lower phosphatization level of prostate cancer cells

Molecular mechanism of busulfan induction of cell apoptosis and lowered survival rate was still not clear. Androgen and its receptors have significant contribution in the occurrence and development of prostate cancer. Androgen receptors can activate protein transcriptions closely relevant to proliferation and apoptosis of cells after binding with ligands as nuclear transcription factor, thus sustain the growth of tumor. Based on this, we conjectured that busulfan may contribute to cell apoptosis via influence over androgen receptors. Firstly, we measured the change in androgen receptor AR mRNA level after 24 h treatment with busulfan of varied concentrations (0 mg/L, 30 mg/L, 60 mg/L, 100 mg/L) and found no significant influence of busulfan concentration on expression of AR (**Figure 3A**). Another test suggested that treatment time have no significant influence on AR expression as no significant difference was found in results of measurement at 0 h, 12 h, 24 h and 48 h after treatment with 60 mg/L busulfan (**Figure 3B**). Thus we conjectured that busulfan may influence translation or post-translational modification since no significant influence on transcription was observed. Results of our later experiment showed that 60 mg/L busulfan inhibited AR phosphatization significantly without influence on expression of AR total protein (**Figure 3C**). We also found that there was a positive correlation between treatment time and decline of AR phosphatization at Y534 site via multiple measurements with intervals (**Figure 3D**).

Busulfan lowered AR phosphatization level via inhibition to Src kinase

We further probed the effect of busulfan treatment on expression level of Ack1 kinase and Src kinase as literal materials reported that Src

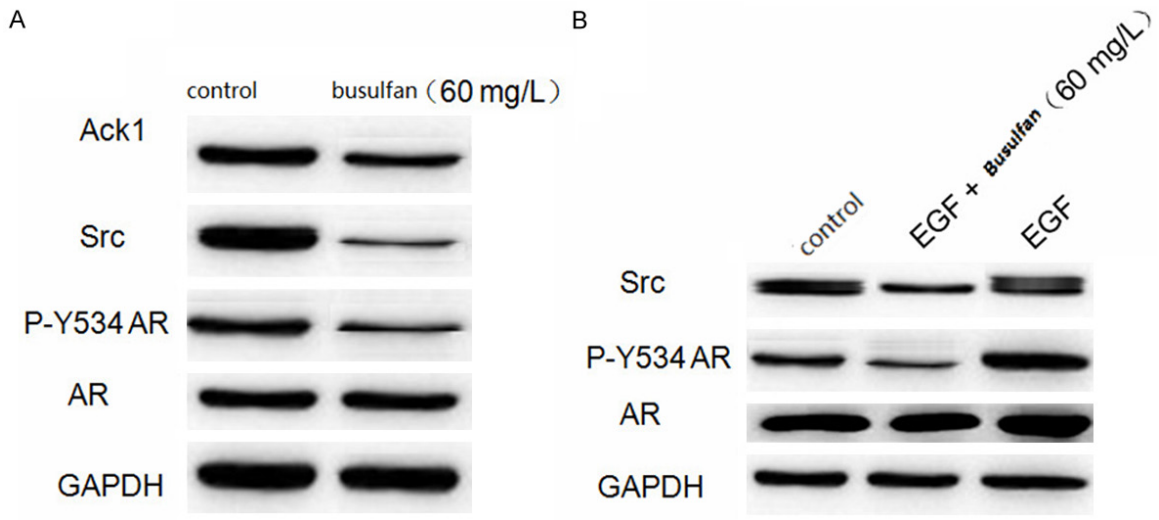

Figure 4. Busulfan lowered AR phosphatization level significantly via inhibition to Src kinase. A. Western blotting probing influence of 60 mg/L busulfan (at 48 h after treatment) on protein expression level of AR, Src and Ack 1 and AR phosphatization level at Y534 site; B. Western blotting probing influence of EGF and busulfan (at 48 h after combination treatment) on protein expression level of AR, Src and Ack 1 and AR phosphatization level at Y534 site.

kinase and Ack1 kinase cause AR phosphatization after EGF treatment on prostate cancer cells. We found slight decline in Ack1 kinase expression level and significant inhibition to expression level of Src kinase, which was identical to the decrease of P-Y534 AR level (**Figure 4A**). Busulfan treatment lowered P-Y534 AR level that was significantly elated by 48 h treatment of 100 ng/ml EGF. Results above suggested that busulfan inhibit AR phosphatization resulting from Src kinase which was induced by EGF (**Figure 4B**).

Busulfan inhibited expression level of androgen receptor downstream target gene PSA and hk2 significantly

Busulfan treatment is inhibitory to phosphatization of androgen receptors, thus lead to restrained AR functions. We measured expression level of AR downstream target gene PSA and hk2 via RT-PCR, and found that expression levels of AR downstream target gene PSA and hk2 declined significantly with the rise of busulfan concentration (**Figure 5A**). Time-dependency of the effect was revealed in later experiments (**Figure 5B**). EGF may facilitate the phosphatization level of androgen receptors by activating Src kinase, contrasting the significant inhibition ability busulfan holds to androgen receptor AR phosphatization. We employed flow cytometry to check whether over-expression of AR downstream target gene PSA and hk2 (caused by adenovirus vector) could resist cell apoptosis caused by busulfan. EGF was used as positive control and found that busulfan held significant inductive power to prostate cancer cell apoptosis, which might be partly remitted by Ad-PSA and Ad-hk2 (**Figure 5D**). In vivo tests showed that EGF, Ad-PSA and Ad-hk2 can partly remit the apoptosis induced by busulfan as significantly earlier appearance and larger size of xenograft were noticed in groups where any of the three were combined to busulfan as compared to single busulfan treatment group (**Figure 5E**).

Discussions

Recent studies revealed the key role of prostate cancer cell AR phosphatization in occurrence of CRPC [20], but its mechanism is still unknown. A study conjectures that extracellular ligands interact with receptors like HER2 and EGFR on the surface of prostate cancer cells, resulting in AR phosphatization via non-receptor kinase signal conduction [21]. Prostate cancer cells treated with EGF may stimulate cellular proliferation under androgen-free condition to a degree close to that under DHT stimulation. There are other reports showing that heregulin [20], bombesin [22] and interleukin 6 [23] also possesses ability to stimulate prostate cancer cell proliferation under androgen-

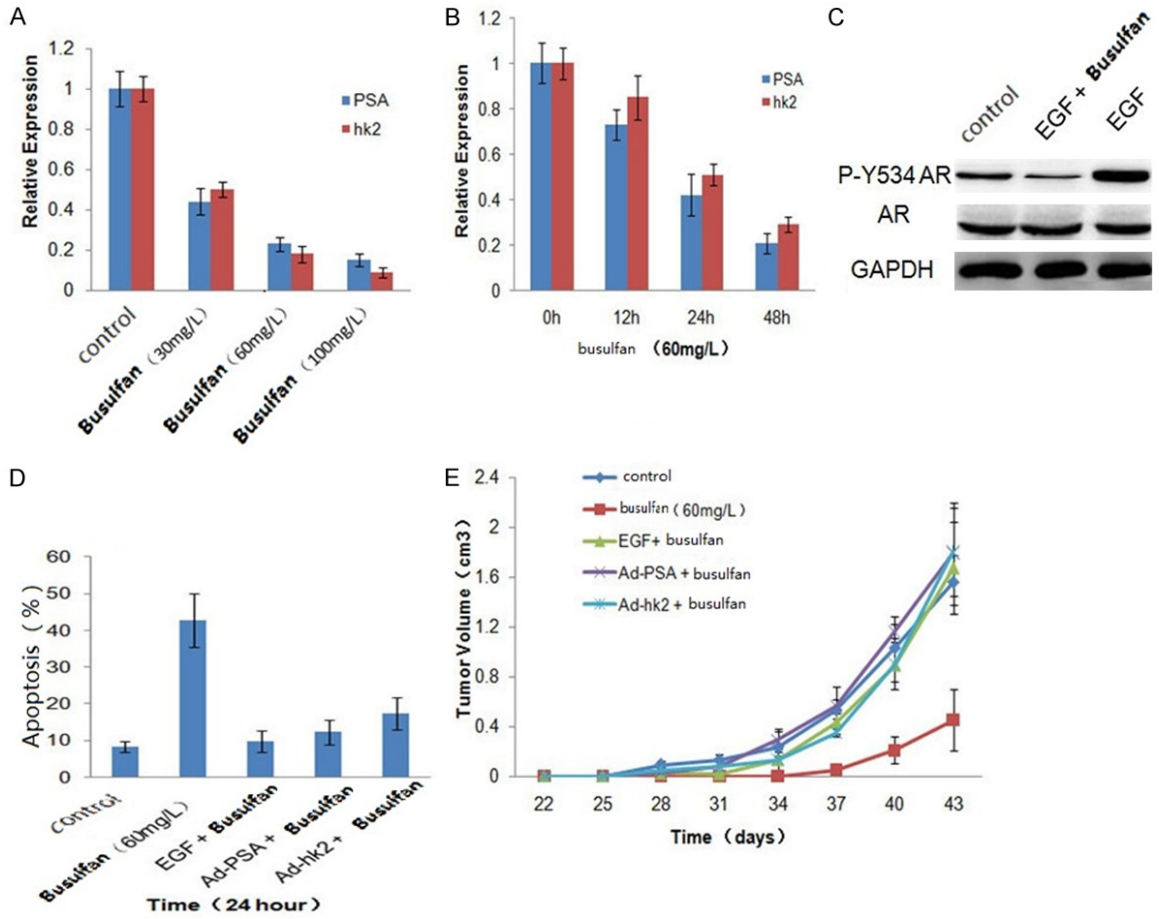

Figure 5. Busulfan may inhibit expression level of androgen receptor downstream target gene PSA and hk2 significantly. A. RT-PCR probing influence of varied concentration (0 mg/L, 30 mg/L, 60 mg/L and 100 mg/L) of busulfan on PSA and hk2 mRNA expression level; B. RT-PC probing influence of 60 mg/L busulfan under time gradient (0 h, 24 h, 48 h, 72 h and 96 h) on PSA and hk2 mRNA expression level; C. Western blotting probing influence EGF and its combination treatment with busulfan on AR expression level and AR phosphatization level at Y534 site at 48 h; D. CCK-8 test probing influences of different treatments (busulfan, EGF+busulfan, Ad-PSA+busulfan, Ad-hk2+busulfan) on cell apoptosis of prostate cancer LAPC4 cells; E. Influence of different treatments on xenograft proliferation ability of prostate cancer cells.

free condition, demonstrating that non-androgen ligands may activate AR, thus facilitates prostate cancer cell proliferation. Expression of AR target gene PSA and hk2, which is closely related to tumor burden may be deemed as tumor marker of prostate cancer [24, 25]. mRNA of PSA and hk2 in prostate cancer cells treated with EGF under androgen-free condition increased significantly than control group, which was in accordance with results of the study.

EGF may, as reported by Liu et al., induce AR phosphatization at Tyr-534 and Tyr-267 site. AR phosphatization at Tyr-534 was inhibited with knockout of Src gene, suggesting that Src kinase contributed to downstream signal pathway of EGF [26], which was in accordance with our results. However, knockout of Ack1 gene, which contributed to AR phosphatization at Tyr-267 site didn't inhibit AR phosphatization there,

suggesting that another pathway exists for AR phosphatization at Try-267 caused by Ack 11 kinase [11]. Kammerer et al. reported that besides cell growth factor, some chemotherapy drugs also adjust the proliferation and apoptosis of prostate cancer cells [27]. Significant decline in prostate cancer cell proliferation ability, as well as significant increase in apoptosis ability, was observed with higher concentration of busulfan or prolonged treatment. We probed molecular mechanism in busulfan inhibition to prostate cancer cell proliferation and found that AR expression level had no significant change in prostate cancer cells treated with busulfan while AR phosphatization level at Try-534 site declined significantly, thus led to significant decline in AR activity. We also found, using RT-PCR, that expression level of PSA and hk2 mRNA declined significantly. Busulfan was significantly inhibitory to androgen receptor phosphatization level since EGF increases it significantly via Src and EGF kinases. Thus we employed flow cytometry to check whether over-expression of AR downstream target gene PSA and hk2 (caused by adenovirus vector) could resist cell apoptosis caused by busulfan. EGF was used as positive control. Results showed that busulfan held significant inductive effect on prostate cancer cell apoptosis, which might be partly remitted by EGF, PSA and Ad-hk2. Our further research on male rats substantiated the conclusion.

Molecular mechanism of busulfan induction of decrease in AR phosphatization was also probed thoroughly in the study. Literal material suggested that Src kinase and Ack1 kinase may cause AR phosphatization under androgen-free condition after prostate cancer cells were treated with EGF [26]. So we detected change in expression of Src and Ack1 kinase after busulfan treatment. Results suggested that expression level of Src kinase decreased significantly on the basis of significant decline in AR phosphatization level at Try-534 site while no significant change in expression level of Ack1 kinase was found. Based on this, we conjecture that busulfan adjusted AR phosphatization via affecting Src activity.

It's not rigorous to simulate the transformation from ADPC to CPRC by depriving androgen from LNCaP cells though it may turn androgen-independent, since variegated changes in gene expression from being passaged in vitro would be missing. Genetic expression and biological behavior of LNCaP cell in generation 20-40 was significantly different to those in generation 80, as was reported by Esquenet et al. [28] and Youm et al. [29] This may affect results of the study since it would take at least 6 months/30-40 generations of culture or the cells wouldn't withstand low androgen condition in the process of in vitro androgen deprivation [30, 31], leaving the mechanism of occurrence and development of prostate cancer under androgen-free condition for future research.

Disclosure of conflict of interest

None.

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