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ESTABLISHMENT AND CHARACTERIZATION OF ABIRATERONE ACETATE RESISTANT PROSTATE CANCER CELL LINE

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ESTABLISHMENT AND CHARACTERIZATION OF ABIRATERONE ACETATE RESISTANT PROSTATE CANCER CELL LINE

Trabalho de Conclusão do Curso de Graduação em Farmácia do Centro de Ciências da Saúde da Universidade Federal de Santa Catarina como requisito parcial para a conclusão do Curso de Graduação em Farmácia.

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RESUMO

O acetato de abiraterona (AA) é um fármaco utilizado para o tratamento de câncer de próstata resistente a castração (CPRC). A introdução do AA na terapia aumentou significativamente a sobrevida dos pacientes com CPRC. Entretanto, o desenvolvimento de resistência ao AA é comum em grande parte dos pacientes. Sendo assim, o desenvolvimento de uma linhagem celular resistente ao AA é útil para a compreensão dos mecanismos moleculares envolvidos neste processo e para o desenvolvimento de novos agentes terapêuticos. A linhagem celular resistente ao AA foi estabelecida a partir da linhagem celular LNCAP após incubações com concentrações crescentes de AA até atingir a IC_{50} da linhagem celular. Posteriormente, foi realizada a confirmação da resistência e a caracterização da linhagem celular. A resistência e a taxa de proliferação celular foram avaliadas pelo ensaio do MTT. A distribuição do ciclo celular e a expressão dos marcadores de células tronco tumorais (CD133 e CD44) foram avaliadas por citometria de fluxo. Em comparação com a linhagem celular parental, a linhagem celular resistente ao AA apresenta a proliferação celular mais lenta e um aumento na resistência de 1,4 vezes. Não foram observadas diferenças em relação a distribuição do ciclo celular, a morfologia celular e a expressão dos marcadores CD133 e CD44 entre a linhagem resistente e a parental. Parâmetros adicionais de caracterização, como expressão específica de miRNAs e expressão da proteína AR-V7, ainda são necessários. Portanto, uma indução de resistência ao AA em uma linhagem de câncer de próstata (LNCAP) foi alcançada, bem como a caracterização da linhagem celular, pelo menos em parte, permitindo estudos adicionais de novos mecanismos de resistência e novas estratégias de tratamento.

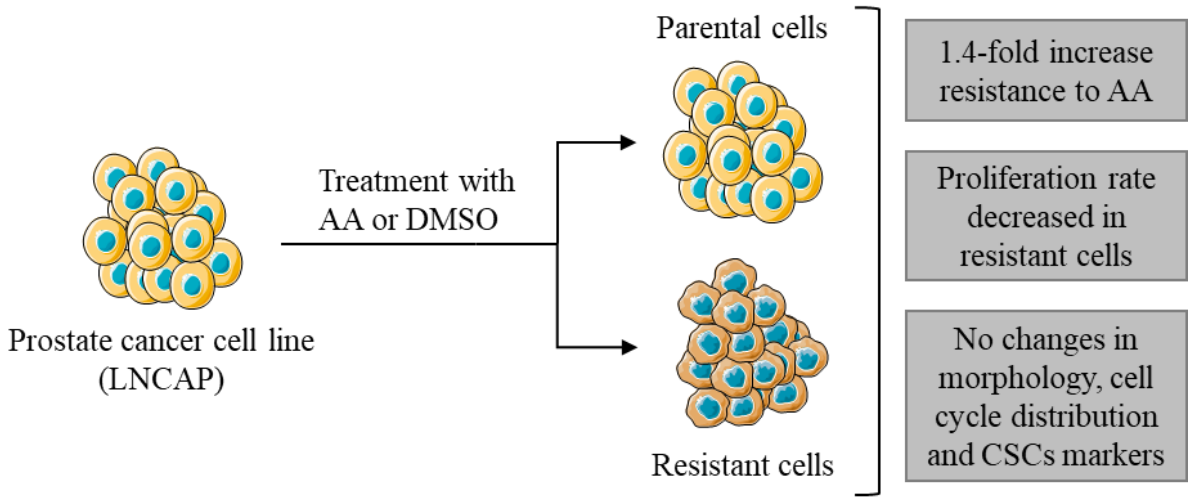
Palavras chaves: câncer de próstata resistente a castração, acetato de abiraterona, resistência a droga, linhagem celular resistente.

ABSTRACT

The abiraterone acetate (AA) is a drug used to castration-resistant prostate cancer (CPRC) treatment. The introduction of AA in therapy significantly increased the survival of patients with CPRC. However, AA resistance development is common in most of the patients. Therefore, the development of an AA-resistant cell line is useful for understanding the molecular mechanisms involved in this process and to the development of novel therapeutic agents. An AA-resistant cell line was established from the LNCAP cell line after incubated with increasing AA concentration until reaches the IC_{50} for the cell line. After that, the resistant confirmation and characterization of the cell line were proceeded. The fold-resistance and cell proliferation rate were performed by MTT assay. Cell cycle distribution and express of cancer stem cells markers (CD133 and CD44) were evaluated by FACS analysis. In comparison to parental cell line, the AA-resistant cell line has a slower growth rate and a 1,4-fold increase in resistance to AA. No differences were observed regarding to cell cycle distribution, cell morphology, and expression of CD133 and CD44 markers between the resistant and parental cell lines. Additional characterization parameters such as specific miRNAs expression and AR-V7 protein expression are still required. Therefore, an AA resistance induction in a prostate cancer cell line (LNCAP) was achieved as well as the cell line characterization, at least in part, allowing further studies of new resistance mechanisms and new treatment strategies.

Keywords: castration-resistant prostate cancer, abiraterone acetate, drug resistance, resistant cell line.

GRAPHICAL ABSTRACT



HIGHLIGHTS

- LNCAP-AAR cell has a 1.4-fold increase in resistance to abiraterone acetate.
- LNCAP-AAR do not show morphological changes when compared to parental cell.
- LNCAP-AAR cell has a slower growth rate than parental cell line.
- LNCAP-C and LNCAP-AAR doesn’t show differences in CSC markers.

SUMMARY

1. Introduction

Prostate cancer is the second most incident cancer in men. In 2018 there are estimated to have been 1.3 million new cases of prostate cancer in the world [1]. The prostate cancer growth is initially responsive to androgen, which means that cell proliferation is dependent on androgenic hormones action. The main circulating androgen in men is testosterone, which is mostly secreted by the testicles, around 80-90%, and about 10-20% in adrenal glands. The testosterone is responsible to bind and activate the androgen receptor (AR), which induces encoding of several proteins that are responsible for prostate cell proliferation and survival [2,3]. Therefore, the initial therapy consists in inhibit the androgens synthesis to reduce the AR activation, and this can be performed by androgen deprivation therapy (ADT). The ADT is effective, but frequently patients undergoing to this therapy do not demonstrate a permanent disease remission and the cancer returns resistant to treatments. When prostate cancer recurrence occurs, the cell proliferation becomes androgen independent and is denominated of castration-resistant prostate cancer (CRPC), which is more lethal and aggressive form of prostate cancer and there is no effective therapy [4,5]. The mechanisms involved to CRPC development are related to changes in androgen receptor (AR) signaling as overexpression, amplification of specificity and activation by growth factors. The CRPC also can be related to malignant epithelial stem cells and the utilization of parallel survival pathways [3].

Therefore, therapy against CRPC involves agents that are more efficient in block androgen synthesis pathways and the abiraterone acetate (AA), a derivative of steroidal progesterone, is one of them. AA is an inhibitor of the androgen synthesis pathway by blocking cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) enzyme, which is present in the testicle, adrenal gland, and prostate. This enzyme is responsible for converting pregnenolone and progesterone into dehydroepiandrosterone (DHEA) and androstenedione, which are testosterone precursors. The drug AA blocks all androgen synthesis pathways, so the reduction in circulating testosterone levels is more significant and then the activity of AR also reduces, decreasing tumor cell proliferation. AR pathway activity reduction is not the only mechanism of AA, the decrease in tumor cells proliferation is also regulated by the modulation of oncogenes and apoptotic pathways [6–8].

However, about 1/3 patients using AA develop primary drug resistance and the mechanisms are still not well evidenced. The possible mechanism used by cells include

androgen synthesis reactivation and/or alterations in AR signaling by genomic variations, as an expression of androgen receptor variant 7 (AR-V7), which shows to be linked to multidrug resistance, including AA [6,9]. Another possibility involved to AA resistance would be related to the presence of a small cell population with similar characteristics to normal stem cells inside the tumors, which are called cancer stem cells (CSC) [10]. The CSCs are responsible for asymmetric divisions, self-renew and tumor heterogeneity, which can promote the chemoresistance and cancer recurrence. Many studies indicate the presence of CSC in CRPC, which are characterized by the high expression of CD133 and CD44 antigens [11–13]. Therefore, the aim of this study was to develop and characterize an AA-resistant prostate cancer cell line with a clinically relevant model for studying mechanisms involved in AA resistance and new treatment strategies in CRPC patients.

2. Materials and methods

2.1. Cell line and culture

The human prostate cancer cell line used was LNCaP, which is responsive to androgen and comes from a metastatic site on the lymph node, has epithelial morphology and adherent properties. LNCaP cell were maintained at 37°C in a 5% CO_2 incubator. The cells were maintained in a cell culture bottle with the appropriate medium for cell nutrition, RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and 10mM HEPES. Culture medium was changed every three days and upon reaching 80% confluency the cells were dissociated with 0.25% trypsin and re-cultivated.

2.2. In vitro development of therapy drug-resistant

The induction of abiraterone acetate resistance by LNCaP cells was performed by using a clinically relevant model [14]. The cells were incubated for three months with increasing AA concentrations (0.5 µM – 18 µM) being denominated as LNCaP-AAR (LNCaP abiraterone acetate resistant). Concomitantly, LNCaP cells was maintained at the same increasing concentration of DMSO, being denominated as LNCaP-C (LNCaP control). After completing the set of incubations, the IC_{50} was determined for both cell lines.

2.3. Cell viability and determination of IC_{50}

Viability of LNCaP-C and LNCAP-AAR cells was evaluated by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [15]. The cells were seeded at the density of 20.000 cells/well in 96 well plates for 24 h and then exposed to abiraterone acetate (10, 20, 30, 40 and 50 µM) for 72 h. After the incubation time the medium was changed by a solution of MTT in medium (0.5 mg/mL) and incubated for 1 h. At the end of incubation time, the medium with MTT was withdrawn and the formazan crystals were dissolved in DMSO, the

absorbance was measured in a spectrophotometer at the wavelength of 540 nm. The results were calculated as a percentage relative to the control (cells treated with DMSO). The concentration required to decrease cell viability at 50% (IC_{50}) was calculated using non-linear regression analysis using software GraphPad Prism 5.0. The cell lines IC_{50} values obtained were used to determine the increase in resistance, known as fold resistance.

$$Fold\ Resistance = IC50\ of\ resistant\ cell\ line \div IC50\ of\ parental\ cell\ line$$

2.4 Cell proliferation

The proliferation rate of the LNCAP-C and LNCAP-AAR cells were evaluated by MTT. The cells were seeded at the density of 20.000 cells/well in 96 well plates and analyzed after 48, 72 and 96 hours by the MTT assay as described in 2.3.

2.5. Cell cycle analysis

The LNCaP sensitive cells were seeded (200.000 cells/well) in 12 well plates and incubated for 24 h then incubated with abiraterone acetate (18µM) for 24 h. After that the cells were dissociated and centrifuged at 300 × g for 5 min. The supernatant was discarded and added an aliquot of 70% cold ethanol and incubated for 20 min at -20°C. After that, an aliquot of PBS plus 2% BSA was added and the cells were centrifuged at 300 × g for 5 min. To the cells pellet was added a solution containing 100µg/mL RNAse + 0.1% Triton-X in PBS. At the time of the analysis, an aliquot of propidium iodide (PI) was added. The analysis was performed on the flow cytometer FACS Canto available in the LAMEB/UFSC and the data analyzed in the Flowing software [16]. The same analysis was realized for the LNCaP-C and LNCaP-AAR cell lines, however the treatment with abiraterone acetate was not performed.

2.6. Quantification of CD133 and CD44 cell surface markers expression

The LNCaP-C and LNCaP-AAR cell lines were seeded (200.000 cells/well) in 12 well plates for 24 h and then the cells were dissociated and fixed with 2% formaldehyde for 20 min. The fixed cells were incubated for 30 min with PBS and 5% FBS for blocking non-specific sites. Then, primary antibodies anti-CD133 and anti-CD44 were added and incubated overnight at 4°C. After incubation, cells were washed with PBS and subsequently incubated with the rabbit anti-IgG secondary antibody conjugated to the Alexa-488 fluorochrome, for 1 h, protected from light. The expression intensity of the CD133 and CD44 markers was analyzed by flow cytometry and the data analyzed using Flowing software. Cells marked only with secondary antibody were used as a negative control.

2.7. Statistical analysis

Data were expressed as means ± standard deviation (SD). The difference between two values was evaluated using the unpaired t-test in GraphPad Prism and $p < 0.05$ was considered statistically significant.

3. Results and discussion

The introduction of abiraterone acetate (AA) and Enzalutamide (androgen receptor-targeted agents - ARTAs) to CRPC therapy significantly improved the patient survival, but the majority of patients develops resistance to these drugs [7,17]. However, the mechanisms involved are not fully understood. It is well established that androgen receptor (AR) mutations are involved in resistance development. Besides inhibition of AR activation, AA also decreases the proliferation of tumor cells by oncogenes modulation and apoptotic pathways [18,19]. Therefore, an AA resistant prostate cancer cell line is required to study and evaluate the mechanisms involved in AA resistance development.

Studies related to AA resistance are scarce and to the best of our knowledge, there is no study using an AA resistant cell line. In this study a prostate cancer cell line resistant to AA was established using LNCAP cells, which is a human prostate cancer cell line androgen responsive, derived from metastatic site in lymph nodes, with epithelial morphology and adherent properties [20]. This cell line was chosen because it is responsive to androgens, so is adequate for evidencing the resistance mechanisms involved with the AR. The induction of AA resistance was performed using a clinically relevant model, in order to mimic the conditions of patients during treatment. Therefore, AA treatment of LNCAP cells was performed with continuous exposure to increasing concentration, initiating with a dose of 0.5µM, which represents the first concentration that patients receive in therapy, until reaching the IC_{50} value for this cell line, 18µM, which was obtained in previous studies (unpublished results). LNCaP cells were incubated for three months with AA or DMSO and two months more without drug for cell proliferation. After this time, the cell viability was analyzed in both cell lines and IC_{50} values were calculated to evaluate the fold resistance. The IC_{50} value for the LNCAP-C cells was 24µM and for LNCAP-AAR cells was 34µM (Fig. 1A). This difference represents 1.4-fold increase in resistance to AA. McDermott et al. [14] reported that the resistance is clinically relevant when a two to five-fold increase is obtained. However, depending on the cell line and treatment performed the fold resistance values may be out of the range provided. As in the studies of Breen et al. [21], Song et al. [22] and Kawai et al. [23], where they reported that in some cell lines the fold resistance was less than two. Each cell line has its own way to respond and adapt to the drug, therefore setting a value range is impracticable.

As shown in fig. 1B, the morphological analysis showed no differences between both cell lines. LNCAP-AAR morphology remained elongated and refringent, similar to parental cells. This similarity was also observed in carboplatin-resistant ovarian cancer cell line, where the morphology in resistant and parental cell lines remained the same [24]. In this article, the authors present an interesting discussion about morphology. In some cell lines when the resistant cells reach differences in morphology when compared to parental cells, they gradually recovered the original morphology characteristics, the changes are probably due to cell stress caused by the drugs.

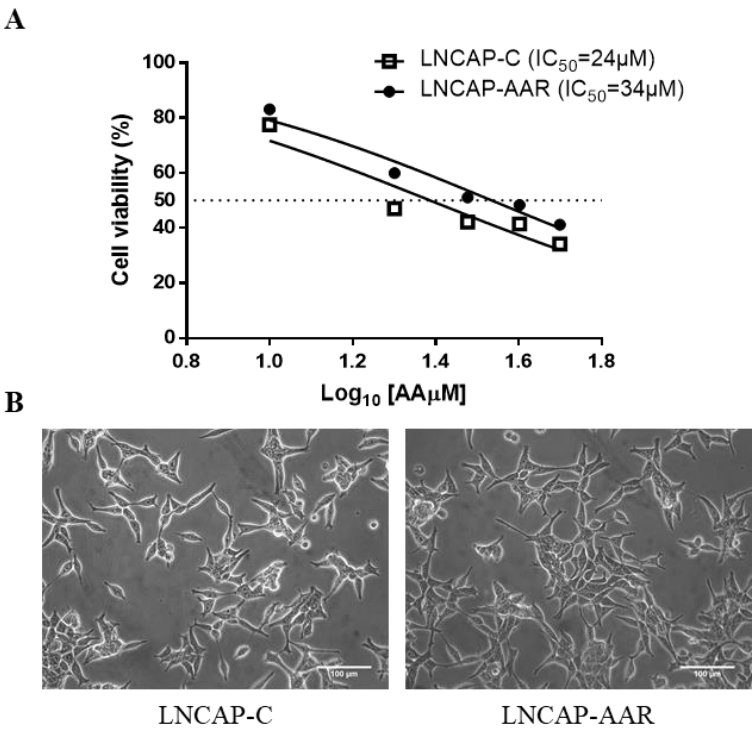


Fig. 1. Cell viability and morphology. (A) Cell viability analysis of LNCAP-C and LNCAP-AAR treated with abiraterone acetate (10, 20, 30, 40 and 50μM) for 72 h was determined by MTT assay, obtaining the IC_{50} values of 24μM for parental cell line and 34μM for the cell line resistant to AA. Values represent the mean (n=2). (B) Cell morphology of LNCAP-C and LNCAP-AAR. Scale bar: 100 μm.

The analysis of proliferation rate of LNCAP-C and LNCAP-AAR demonstrated that LNCAP-AAR has a slower rate of proliferation when compared to parental cells (Fig. 2A). This characteristic is in accordance with the study of Riahi-Chebbi et al [25], where the 5-Fluorouracil resistant colon cancer cell line has a slower growth compared to the parental cells. The proliferation data in 96 h do not show differences, probably because the confluence of the LNCAP-C cells. Studies suggest that CYP17A1 enzyme inhibition is not the only AA

mechanism and that AA anti-tumor activities are related to regulatory factors, apoptosis, and cell cycle pathways [7]. Therefore, we analyzed the AA effect in cell cycle of LNCAP cells to see how the drug acts on cell cycle. It was observed that cells arrest in phase G0/G1, followed by reduction on phase S and phase G2/M (Fig. 2B), which means that AA reduces the tumor cells proliferation by changes in cell cycle distribution. When the cell cycle in LNCAP-C and LNCAP-AAR without treatment was analyzed no differences in cell cycle distribution (Fig. 2C) were observed.

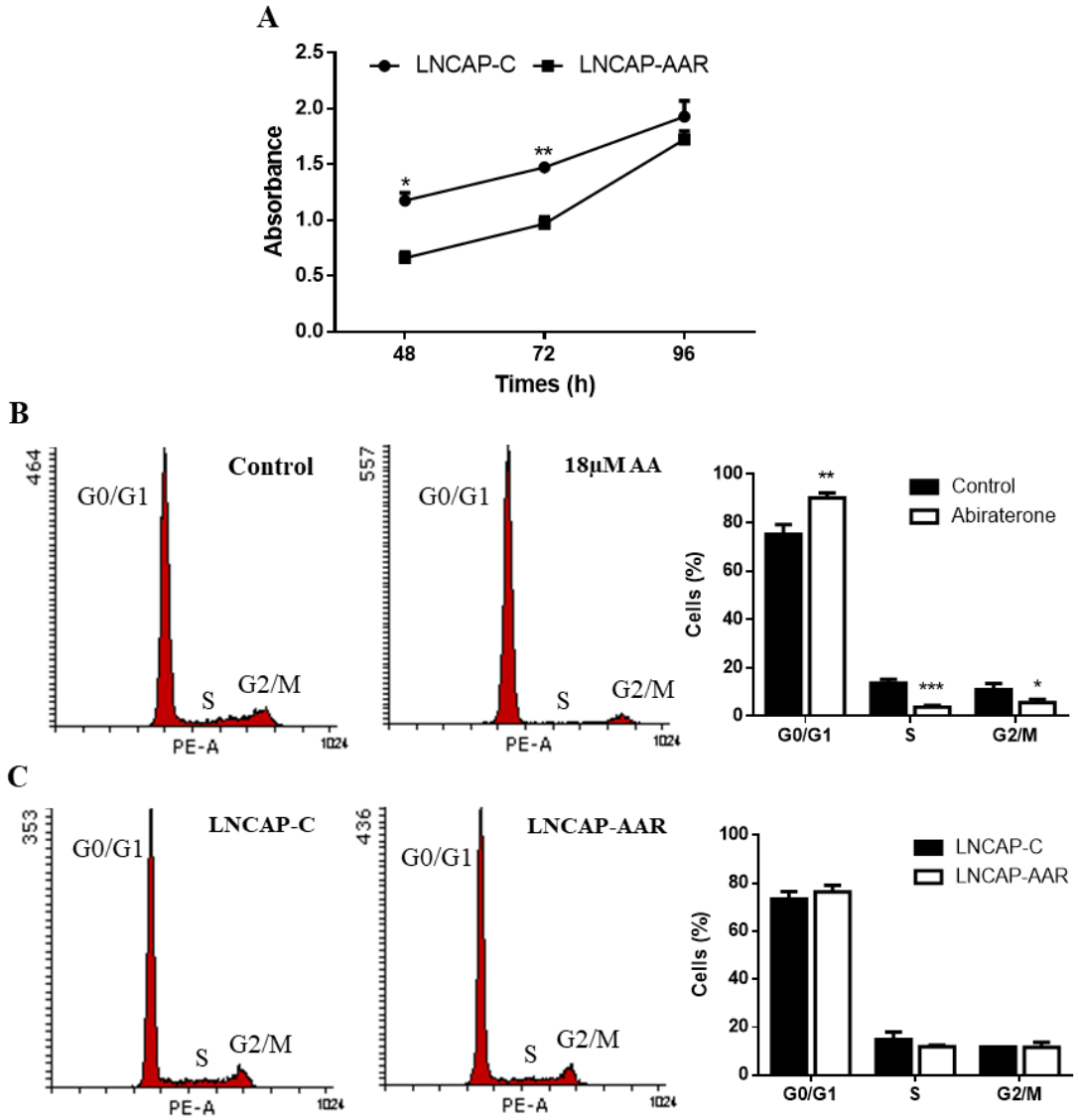


Fig. 2. Cell proliferation and cell cycle analysis. (A) Proliferation rate of LNCAP-C and LNCAP-AAR cells for 48 h, 72 h and 96 h determined by MTT assay. (B) Distribution of sensitive cells in different cell cycle phases treated with abiraterone acetate 18 µM compared to the control (treated with DMSO). (C) Distribution of LNCAP-AAR and LNCAP-C in different cell cycle phases. Values represent the mean ± standard error (n=2). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

In a study realized by Lypova et al. [26] they analyzed the resistant and the parental cell lines with and without treatment, to see if the drug would still have effect in cell cycle after acquiring resistance. Therefore, they saw that the treatment had no effect on cell cycle distribution in the resistant cell line. This effect can be analyzed in further studies in order to check if the LNCAP-AAR is less susceptible to the cell cycle effects of AA.

There are strong evidences demonstrating the presence of cancer steam cells (CSC) in CRPC and the relation with drug resistance [13]. So the enrichment in stem cell markers (CD133 and CD44) supports the presence of a chemoresistant population [27]. The expression of CD133 antigen is correlated to a high cell growth potential, to regenerate a differentiated prostatic epithelium and higher clonogenic potential [11], and the cell expression of CD44 antigen is correlated to several biological processes like cell adhesion, metastasis and tumor invasion [28]. For that reason, the expression of prostatic CSCs markers CD133 and CD44 was analyzed in both cell lines. Our results showed that LNCAP-C and LNCAP-AAR do not show differences in antigens cell expression (Fig. 3A and B). Similarly, Barr et al. [27] showed that some of Cisplatin-resistant lung cancer cell lines do not express differences in CSC markers when compared to the parental cell line.

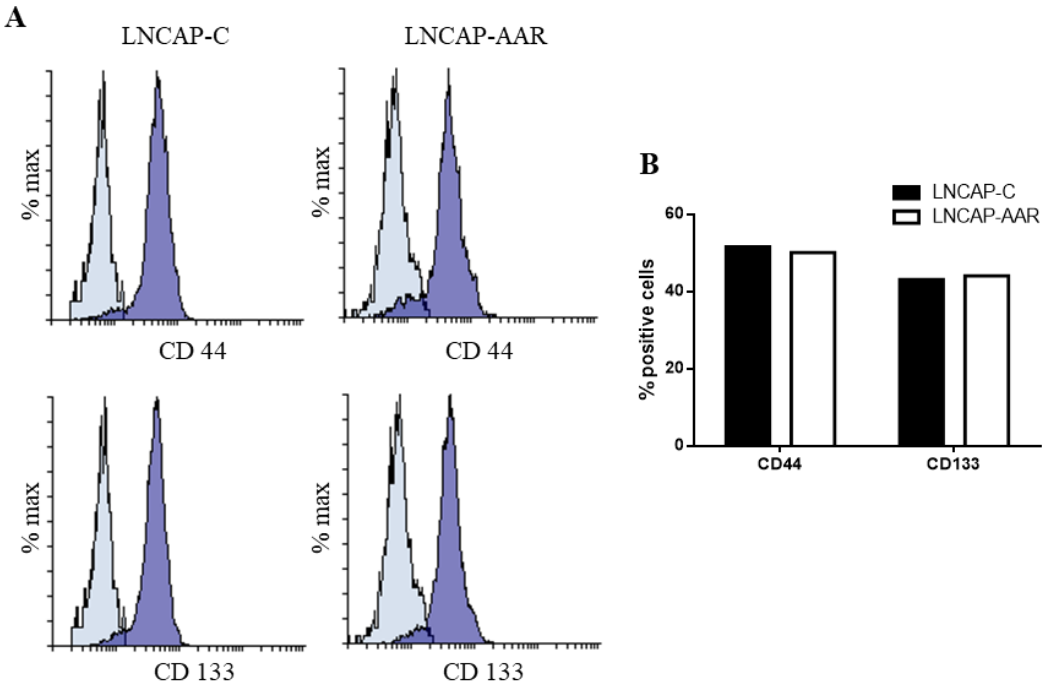


Fig. 3. Expression of CD133 and CD44 markers. (A) Expression analysis of CD44 and CD133 markers in LNCAP-AAR and LNCAP-C cells by flow cytometry using Alexa-488 fluorochrome. The light blue histogram is the negative control. (B) Columns represent the percent of positive cells for each marker.

The detection of genomic variation AR-V7 in circulating tumor cells appears to be related with resistance to abiraterone and enzalutamide and can be used as a biomarker to predict resistance in patients [29]. Therefore, AR-V7 detection in LNCAP-AAR cells would confirm the AA resistance. The expression of variation AR-V7 in LNCaP-AAR will be useful for study new therapies. For example, the enzalutamide is an androgen receptor-targeted agent and is an option to CRPC treatment, as the AA. When the patients develop AA resistance, they also became enzalutamide resistant, and the reciprocity is true. Therefore, as already was demonstrated that Niclosamide, a potent inhibitor of AR-V7 protein expression, with enzalutamide treatment can turn resistant tumor cells re-sensitize to enzalutamide and improve the efficacy of enzalutamide therapy [30]. So, the treatment with niclosamide in combination with AA could be performed in LNCAP-AAR in order to check if the re-sensitization also occurs in AA resistance. Furthermore, Detassis et al. [31] showed that the miR-103a-3p and miR-378a-5p are able to identify respondent patients to AA treatment. These miRNAs can be analyzed in further studies in order to check the relation of these molecules and the resistance mechanisms in LNCAP-AAR.

In summary, our study is the first to report the development and characterization of an AA-resistant prostate cancer cell line. However, further studies should be performed to identify the mechanisms that confer resistance to LNCAP-AAR cells, such as specific miRNAS expression and AR-V7 protein expression. The resistant cells exhibit 1.4-fold increase resistance to AA, compared to their parental cells. In addition, the growth rate decreased in resistant cells, without changes in morphology, cell cycle distribution, and CSCs markers. The established cell line is important to denote a clinically relevant *in vitro* model to study the molecular mechanisms involved with AA resistance development and for identify new drug for overcoming such process.

ACKNOWLEDGMENTS

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