CONTRIBUTION OF EXPRESSION OF C-KIT IN THE AQUISITION OF CHEMORESISTANCE TO DOCETAXEL IN PROSTATE CANCER

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ABSTRACT

Background: castration-resistant metastatic prostate tumors respond poorly to chemotherapeutic treatments. The bone microenvironment induces certain genetic alterations in prostate cancer cells, such as the c-KIT expression. Since the association of the c-KIT expression is linked to the development of chemoresistance in other tumors and the outcome of the chemotherapeutic treatment of prostate cancer with bone metastases has poor results, we hypothesized that the induction of the c-KIT expression in prostate cancer with metastases in bone tissue contributes to the reduced sensitivity of prostatic neoplasia to Docetaxel.

Objective: evaluating the hypothesis that the c-KIT expression by prostate cancer cells contributes to the reduced sensibility of this neoplasia to chemotherapeutic agent Docetaxel.

Materials and Methods: previously c-KIT negative PC3 and C42b cells underwent c-KIT cell transfection. These cells were treated with doses of Docetaxel and, at 48 or 72 hours of treatment, were subjected to colorimetric WST-1 assay.

Results: we observed that in the experiments treated with Docetaxel for 2 days, the IC50 values of the groups C42b c-KIT and PC3 c-KIT were superior to the IC50 values of the groups C42b and PC3 that had not undergone the stable c-KIT transfection ($p<0.001$ groupxdose). In experiments treated with Docetaxel for 3 days, we did not observe any statistically significant difference of IC50 values between the groups of C42b cells transfected or not transfected with c-KIT and PC3 cells.

Conclusions: The present study showed no increased chemoresistance of C42b and PC3 cells to the chemotherapeutic agent Docetaxel when they are transfected with c-KIT.

Key Words: Prostate Cancer, Bone metastasis, C-KIT, Docetaxel

INTRODUCTION

With the exception of non-melanoma skin tumors, prostate cancer is the most commonly diagnosed malignant tumor among men, with approximately two hundred and seventeen thousand new cases per year in the United States, causing approximately thirty thousand deaths annually in this country. It is the second greatest cause of death of cancer in the Western male population (1). Based on autopsy study, about 90 % of men who die of prostate cancer have bone metastases (2).

The treatment for the metastatic prostate disease is palliative. The androgen ablation is the initial treatment for patients with metastases. However, this therapy will fail for most men, and they will progress into the stage called hormone-refractory or castration-resistant tumor (3), which is regarded as the tumor that progresses regardless of the androgen deprivation therapy. The progression may be present in any combination of PSA elevation, progression of the preexisting disease and appearance of new metastases.

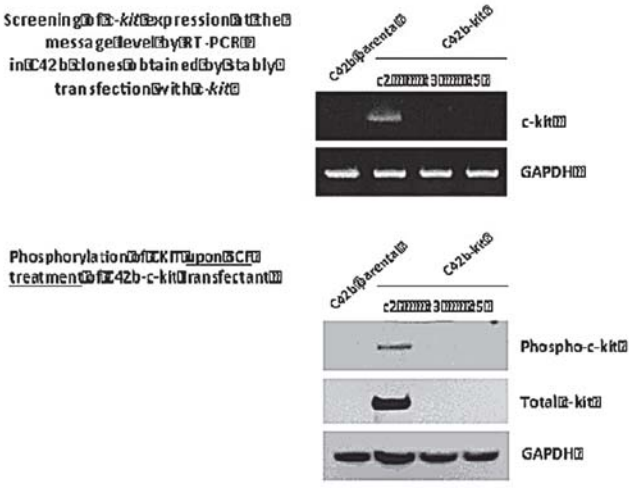
The standard treatment for hormone tumors resistant to androgen ablation is the combination of Docetaxel with prednisone. Docetaxel is an antitumor agent of the taxane family, which acts as an inhibitor of cellular functions like cell division and is used in other tumors, such as breast and lung tumors. Docetaxel, when compared to Mitoxantrone, considered a firstline treatment until 2004, was superior in terms of improving pain standards, decreasing PSA, improving quality of life and increasing survival rates (4, 5). The bone microenvironment, through a bidirectional interaction with metastatic prostate cancer cells, causes phenotypic alterations in the cells. One of these alterations is the expression of previously c-KIT negative metastatic cancerous cells of c-KIT receptors (6). Some tumors, such as GIST (gastrointestinal stromal tumor), acute myeloid leukemia (7,8), small-cell lung cancer (9), breast cancer (10) and mesothelioma (11), showed abnormal c- KIT and/or SCF expression and activation, leading to increased proliferation and to decreased tumor apoptosis. Numerous clinical studies have indicated that increased c-KIT expression and/or c-KIT mutation are strongly associated with decreased survival rates and poor prognosis for some tumors (12). Based on the poor response of metastatic prostate tumors to chemotherapeutic agents, on the phenotypic alterations of prostate cells, expressing c-KIT when in contact with the bone microenvironment, and on the association of this cellular alteration with the worst prognosis in other tumors, we hypothesize that the c-KIT expression in prostate cancer cells contributes to the chemoresistance of such tumors.

MATERIALS AND METHODS

Cell culture: we used PC3 and C42b cells in this study, which are originally derived from bone metastasis of androgen-independent human prostate cancer (13) and originated in lumbar spine bone metastases in athymic mice (14), respectively, obtained originally from the American Type Culture Collection (Manassas, VA). These cells were cultured in the culture medium RMPI 1640 (Invitrogen, Carlsbad, CA), with 10% fetal bovine serum (FBS, Atlanta_Biologicals, Lawrence-Ville, GA), 100 IE/ml penicillin and 100 U/ml streptomycin, and incubated at 37° C and 5% CO2. Cell transfection: the technique was used in order to mimic the c-KIT expression *in vitro*, so as to simulate what is observed when prostate cancer cells are in contact with the bone microenvironment. c-KIT negative PC3 and C42b cells were subjected to stable c-KIT transfection. The cell lines used were cultured one day before the transfection, in a number of 1 x 105 cells of each line, in 500 of growth medium in a 6-well titer plate, without antibiotics, so that cells reached a 90-95% confluence at

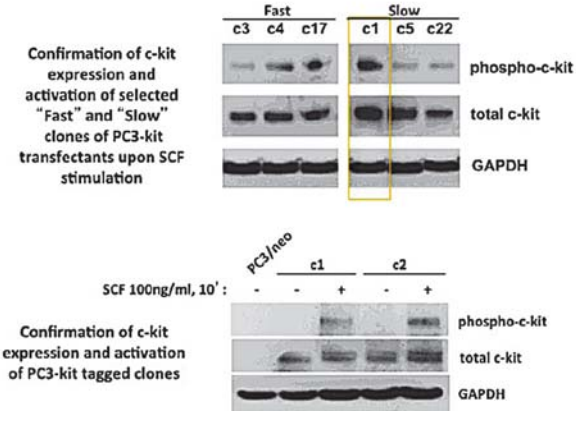
the time of the transfection. On the following day, 8 (4) of the plasmid were diluted in 250 of Opti-MEM®, mixing gently. The incubation was carried out for 5 minutes at room temperature. Subsequently, the diluted plasmid was combined with LipofectamineTM 2000, also diluted, mixing gently for 25 minutes at room temperature (negative control were cells cultivated in 2.5 ml of RPMI 1640 with 10% SFB) . For each culture well, 100 of this solution were used. The cells were taken to the CO2 incubator and incubated at 37° C and 5% CO2 for 48 hours. The culture medium was changed every 6 hours from the start of incubation. After the first 48 hours, the cells were split using trypsin and transferred to a 25 negative control were cells cultivated in 2,5 ml of RPMI 1640 with 10% SFBcm flask. At this moment, the treatment with G418 at the concentration of 350 /ml was initiated. The cells were grown in this medium until the ones in the control group had died by using the same medium (with G418). The type of plasmid used was pCMV6-NEO (cat. SC120061 in pCMV6-NEO). This vector is resistant to Geneticin. Selection of transfectable clones: several clones were selected and the protein expression was confirmed by RT-PCR (Reverse-Time Polymerase Chain Reaction) and Western Blot. The cells transfected with c-KIT were expanded 28 days after the beginning of the treatment with G418 and cultivated in 75 square cm flasks (4 x 103 cells/flask) in complete medium without G418. When colony growth became visible, circles were drawn around them outside the flasks, using highlighters. The areas were collected with cotton swabs using trypsin. The cells were transferred to a 24-well titer plate with 1.5 ml of complete cell medium. When they expanded, they went into a 25 square cm flask, and, whenever possible, into a 75 square cm flask. From then on, the cells were treated with G418 again. When they reached 90% confluence, they were divided and separated by cell clones (clone number 1, number 2, etc.) once more. RT-PCR: used to evaluate the cells transfected with c-KIT (figure 1, figure 2).

FIGURE 1
SCREENING OF THE C-KIT EXPRESSION IN C42B CELLS, BY RT-PCR IN THE CLONES OBTAINED BY STABLE C-KIT TRANSFECTION.



Fonte: Arquivo pessoal

FIGURE 2:
SCREENING OF THE C-KIT EXPRESSION IN PC3 CELLS, BY RT-PCR IN THE CLONES OBTAINED BY STABLE C-KIT TRANSFECTION.



Fonte: Arquivo pessoal

To this end, the two cell lines were cultured in 100% confluence in a 24-well titer plate. These cells were subjected to RNA extraction, using the reagent Trizol® (Invitrogen, Cat. n. 15596-026). When the RNA was resuspended, we used IScript cDNA Synthesis Kit (BioRad cat. n. 1708890), following the kit's technical standards, for the spectrophotometric analysis with BioRad Smart Spec 3000. Shortly after, we conducted the reverse transcription in the GeneAmp PCR and, using the same equipment, the PCR reaction, using the proper primers. As the PCR was completed, the agarose E-gel cassette was loaded with samples from eppendorf tubes and loaded into the E-Gel IBase Power System. After this step, the gel was ready to be used in the UV transilluminator. Western Blot: The protein analysis was performed using the Western blot technique, both in the cells that were or were not transfected with c-KIT. Cells treated with the antagonists were placed in lysis buffer and subsequently homogenized in cold RIPA buffer containing protease inhibitors, centrifuged at 200 g for 5 minutes, resuspended in 1 mL of buffer in conical tubes, centrifuged at 600 g for 10 minutes at 4° C. We collected the centrifugation supernatant, determined the protein concentration and stored it in 50 µL aliquots. The protein concentration present in the cultures was determined using 660 nm Protein Assay (Thermo Scientific). During the procedure, 150 µL of the reagent were added to the 10 µL of the sample that was going to be dosed, according to the manufacturer's specifications. The absorbance intensity of the resulting mixture was determined at 660 nm, after 10 minutes of reaction. Polyacrylamide / SDS Gel Electrophoresis:We resuspended 50 µg protein aliquots in 30 µL of sample buffer [Tris-HCL 500 mM, pH 6.8, glycerol, 10% SDS (sodium dodecyl sulfate), β-mercaptoethanol, 0.05% bromophenol blue]. Then, the samples were heated in a dry bath at 95° C for 10 minutes. The total volume of the samples, as well as 10 µL of standard molecular weight for protein, were subjected to electrophoresis, applied in 12% polyacrylamide gel, at a voltage of 80 V for around 2 hours, until the proteins reached the separating gel. When the voltage reached 120 V, the proteins were separated according to their molecular weights, having the standard as a parameter. Western Blotting: After performing SDS-PAGE as described above, we performed the immunoblotting through the electrophoretic transfer (250 mA, for 1 hour) of the proteins from the polyacrylamide gel to a nitrocellulose membrane. The membrane was incubated with blocking solution 7.5% skimmed milk in Tris 200 mM buffer, pH 7.5, containing 500 mM NaCl, for 30 minutes, before the 12-hour incubation at 4° C, with the primary monoclonal antibody, whose dilutions were prepared according to the manufacturer's specifications, restricted to the respective proteins (1:500). Successive PBS washes were performed to remove non-specific bindings; the membrane was incubated with the secondary antibody conjugated to peroxidase, for 1 hour. The immunoreactive bands were visualized with the chemiluminescence reagent Renaissance (NEN Life Science Products), proceeding according to the manufacturer. Treatment of cells with Docetaxel: PC3 C42B c-KIT positive and c-KIT negative cells were cultured at a concentration of 5 x 103 cells/ml for C42B in each cell of 96-well titer plate and 2.5 x 103 for PC3 in each cell of 96-well titer plate the day before the experiment, quadrupled. One day after culturingthe cells, half of each of the groups (C42B c-KIT, C42B, PC3 c-KIT, PC3) received the treatment of 100 ng/ml SCF. After one hour of treatment with the ligand, the treatment with increasing doses of Docetaxel (Sigma-Aldrich, St. Louis, MO) started, for each cell group with or without c-KIT and activated or not activated with SCF. Docetaxel was stored at -20° C and was dissolved in 1 ml of DMSO before use, with a final concentration of 1 mg/ml for stock. Cell chemosensitivity assay: the colorimetric WST-1 assay was carried out 48 or 72 hours after the beginning of the treatment with Docetaxel. In order to do this, we followed the manufacturer's protocol (Cell Proliferation Reagent WST- 1, ROCHE Applied Science, catalog number 1 644 807). Statistical analysis: The average absorption was calculated for each cell group and for each cell variation. Using this average, we calculated the inhibition rate by subtracting the average absorption value at 100%. The cell vitality inhibition rates were converted

to probits (probability units) and the doses of Docetaxel received logarithmic transformation. After that, we used covariance analysis to compare the groups and identify differences between groups, using Sidak post-hoc test. To obtain the IC50, value, we used the formula IC50 = 10((5-b0)/b1) from the probits of the covariance analysis. To draw the chart lines we used the parameters of linear equations of each group obtained in the covariance analysis followed by a retro-transformation of probits into percentages. To this end, we used SPSS 21 and Sigma Plot 11.

RESULTS

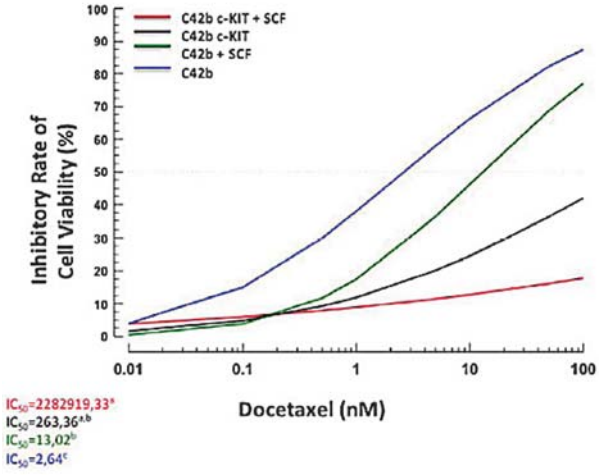
The experiments were divided into days of treatment with Docetaxel and into different doses of Docetaxel. The main parameter for each experiment was the half maximal inhibitory concentration (IC50). According to the FDA (Food and Drug Administration), the IC50 value represents the drug concentration required for 50% of *in vitro* cell inhibition (15). In the experiment shown in figure 3, the C42b cells were cultured 5 x 103 cells/ml and the treatment with Docetaxel was carried out for 48 hours. The final concentrations of Docetaxel were 0, 0.1, 0.5, 1, 10, 50, 100 (nM). The IC50 values for this experiment were C42b 2.64, C42b + SCF 13.02, C42b c-KIT 262.36, C42b c-KIT + SCF 2282919.33 (P group x dose < 0.001) (figure 3). In the experiment shown in figure the C42b cells were cultured 5 x 103 cells/ml and the treatment with Docetaxel was conducted for 72 hours. The final concentrations of Docetaxel for this study were 0, 0.1, 0.5, 1, 10, 50, 100 (nM). The IC50 values for this experiment were C42b 0.98, C42b + SCF 0.95, C42b c-KIT 3.94, C42b c-KIT + SCF 2.62 (P group x dose = 0.052) (figure 4). Since there was a significant decrease of the difference in the IC50 values between the groups when the treatment was carried out for 72 hours, we conducted a new experiment (figure 5) using doses of Docetaxel similar to the IC50 found in the previous experiment **(figure 4)** (0, 0.1, 0.5, 1, 2, 4, 5, 25 (nM)). Again, the IC50 values for the groups were C42b 0.53, C42b + SCF 0.60, C42b c-KIT 0.67, C42b c-KIT + SCF 0.54 (P group x dose = 0.043) (figure 5). We performed the previous experiment once again, using the same doses of Docetaxel, for 48 hours (figure 6). The results were C42b 1.45, C42b + SCF 1.38, C42b c-KIT 5.35, C42b c-KIT + SCF 6.24 (P group x dose = 0.08). For PC3 cells we performed the culture of 2.5 x 103 cells/ml with doses of Docetaxel at 0, 0.1, 0.5, 1, 5, 10, 50, 100 (nM) for 48 hours (figure 7). The results of the IC50 values found were PC3 44.06, PC3 + SCF 39.40, PC3 c- KIT 7.28, PC3 c-KIT + SCF 4514289.14 (P group x dose < 0.02). Similarly to what we did with C42b cells, the treatment was extended, in a new experiment for 72 hours, using the same doses of Docetaxel (0, 0.1, 0.5, 1, 5, 10, 50, 100 (nM)) (figure 8). The results of the IC50 values were PC3 11.01, PC3 + SCF 8.54, PC3 c-KIT 5.10, PC3 c-KIT + SCF 8.13 (P group x dose = 0.384). In an attempt to understand the increased chemoresistance to the treatment with Docetaxel for 3 days in the groups without c-KIT transfection, we performed the cell doubling time in the groups C42b and PC3, which also showed no difference between groups (PC3 EV DT = 40 hours, PC3 c-KIT DT = 32 hours, C42b EV DT = 12 hours, C42b c-KIT DT = 14 hours) (Figures 9, 10).

DISCUSSION

The results we presented here reveal interesting aspects of the role of c-KIT expression by prostate cancer cells when in contact with the bone microenvironment. The analysis we carried out here is that the c-KIT expression by these cells would increase the chemoresistance to the treatment with Docetaxel, based on several articles that demonstrate that some tumors (6, 7) showed abnormal c-KIT and/or SCF expression and activation, leading to increased proliferation and to decreased tumor apoptosis. This guided other clinical studies to show that increased c-KIT expression and/or c-KIT mutation were strongly associated with decreased survival rates and poor prognosis for some tumors (12). The initial results of this study, using doses greater than 10 nM of final concentration of Docetaxel, treated for 48 hours, showed an increase of the IC50 values for the groups that underwent c-KIT transfection, regardless of the treatment with the ligand SCF, statistically significant (P < 0.001) and with a great difference between the IC50 values of the groups (the IC50 values found for PC3 and C42b was > 100 for c-KIT groups). Nonetheless, when we performed the cell treatment with Docetaxel for 72 hours and we approached the doses of Docetaxel near the IC50 values found earlier (between 3 and 5) , we found IC50 values that are lower and are no different between groups that are and are not transfected with c-KIT.

FIGURE 3

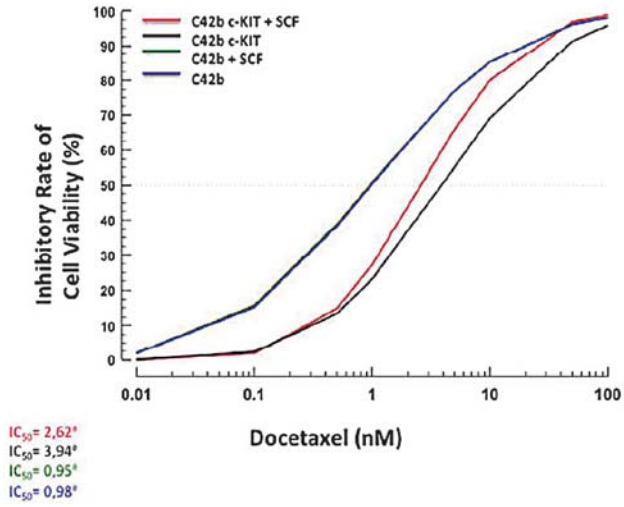
C42b, number of cultured cells = 5 x 103 cells/ml, 48 hours of treatment with Docetaxel in the final doses of 0; 0.1; 0.5; 1; 10; 50; 100 (nM).IC50 C42b 2.64 C42b + SCF 13.02 C42b c-KIT 263



Fonte: Arquivo pessoal

FIGURE 4

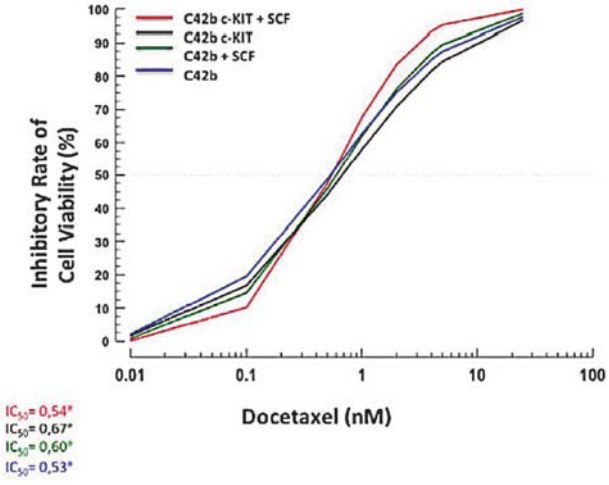
C42b, number of cultured cells = 5 x 103 cells/ml, 48 hours of treatment with Docetaxel in the final doses of 0; 0.1; 0.5; 1; 10; 50; 100 (nM). IC50 C42b 0.98 C42b + SCF 0.95 C42b c-KIT 3.94



Fonte: Arquivo pessoal

FIGURE 5

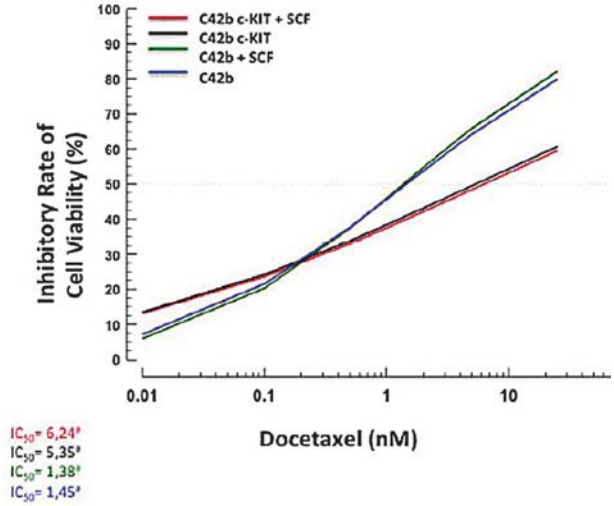
C42b, number of cultured cells = 5 x 103 cells/ml, 72 hours of treatment with Docetaxel in the final doses of 0; 0.1; 0.5; 1; 2; 4; 5; 25 (nM). IC50 C42b 0.53 C42b + SCF 0.60 C42b c-KIT 0.67



Fonte: Arquivo pessoal

FIGURE 6

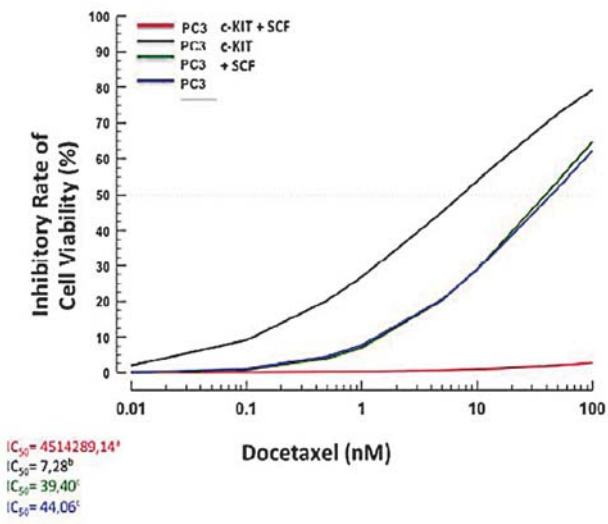
C42b, number of cultured cells = 5 x 103 cells/ml, 48 hours of treatment with Docetaxel in the final doses of 0; 0.1; 0.5; 1; 2; 4; 5; 25 (nM). IC50 C42b 1.45 C42b +SCF 1.38 C42b c-KIT 5.35



Fonte: Arquivo pessoal

FIGURE 7

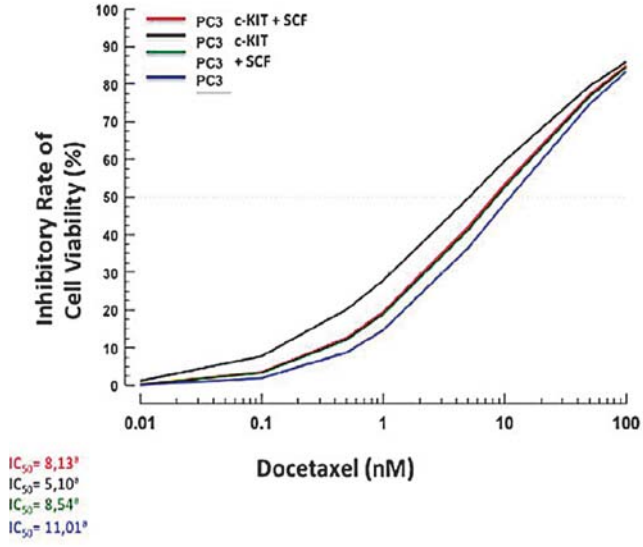
PC3, number of cultured cells = 2.5 x 103 cells/ml, 48 hours of treatment with Docetaxel in the final doses of 0; 0.1; 0.5; 1; 10; 50; 100 (nM).IC50 PC3 44.06 PC3 + SCF 39.40 PC3 c-KIT 7.28



Fonte: Arquivo pessoal

FIGURE 8

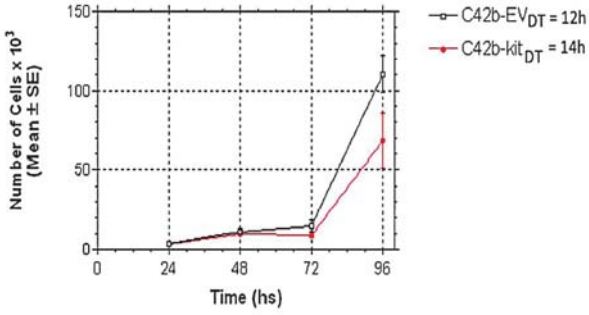
PC3, number of cultured cells = 2.5 x 103 cells/ml, 72 hours of treatment with Docetaxel in the final doses of 0; 0.1; 0.5; 1; 10; 50; 100 (nM).IC50 PC3 11.01 PC3 + SCF 8.54 PC3 c-KIT 5.10



Fonte: Arquivo pessoal

FIGURE 9

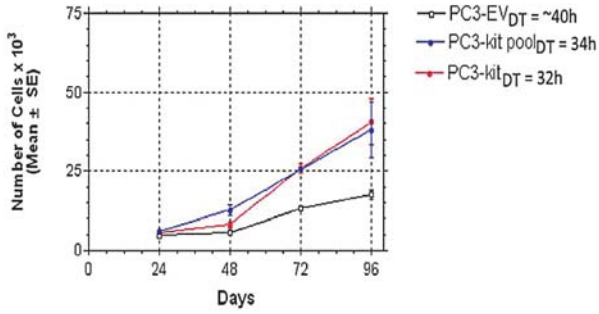
Doubling time for C42b - EV and C42b c-KIT



Fonte: Arquivo pessoal

FIGURE 10

Doubling time for PC3 - EV and PC3 c-KIT



Fonte: Arquivo pessoal

CONCLUSIONS

Even though several studies show a more aggressive tumor, resistant to the available chemotherapeutic treatments, when in the presence of c-KIT activation, this study has not demonstrated an increase in cell chemoresistance when they are transfected with c-kit. Further studies should be conducted to clarify the role of c-KIT in prostate cancer cells when in contact with the bone microenvironment.

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