

C-MYC modulation induces responsiveness to paclitaxel in adrenocortical cancer cell lines

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Abstract. C-MYC is overexpressed in many types of cancer linked to poor prognosis. We examined the c-Myc protein expression in adrenocortical cancer (ACC) cells to investigate the role of this protein in the neoplasm, its involvement in chemotherapy and finally to determine whether c-Myc could be considered a prognostic factor in patients with ACC. H295R and SW13 cell lines were treated with paclitaxel. c-Myc overexpressing cell clones were achieved by transfecting the H295R cell line with the pcDNA3-hMYC plasmid expressing the full-length C-MYC coding sequence. The SW13 cell line was transfected with siRNA oligonucleotides for C-MYC. Cell cycle analysis was evaluated by flow cytometry. c-Myc, cyclin B1 and pro caspase expression levels were evaluated by western blot analysis. We found that expression of c-Myc was highly expressed in the SW13 cells, whereas the protein was undetectable in the H295R cells. Different doses of paclitaxel were required in the two ACC cell line to induce a block in the G₂ phase, characterized by increased cyclin B1 levels and to induce apoptosis by pro-caspase-3 activation. Interestingly, the silencing of C-MYC mRNA prevented paclitaxel induced apoptosis in SW13 cells, whereas in the H295R cells the overexpression of C-MYC rendered the cells more prone to growth inhibition after paclitaxel exposure. The present study directly demonstrates that C-MYC plays a central role in controlling proliferation in ACC cells after paclitaxel treatment and that c-Myc could be considered as a marker for predicting response to chemotherapeutic agents in ACC cell lines.

Introduction

Adrenocortical carcinoma (ACC) is a rare endocrine neoplasia with a variable prognosis, depending on tumor stage and time of diagnosis, but it is generally fatal, with an overall survival of 5 years from detection (1-3). Metastasis associated with ACCs can range from 30 to 85% (4).

More recently, elevated or deregulated expression of the c-Myc protein has been detected in a wide range of human cancers, and is often associated with aggressive and poorly differentiated tumors. C-MYC belongs to the class of immediate early genes which are induced in response to a number of mitogenic signals. It encodes a nuclear transcription factor leading to activation or repression of target genes, thus affecting several biological processes, such as cellular growth, differentiation, cell cycle and apoptosis (5,6).

Surprisingly, gene expression profile studies have demonstrated an underexpression of C-MYC in ACC compared to adrenocortical adenoma (7-10).

The treatment with DNA-damaging agents can sensitize cells to apoptosis when c-Myc is overexpressed (11), but its role in cellular susceptibility to anticancer drugs is controversial. It has been reported that the overexpression of c-Myc not only enhances tumor cell sensitivity (12) but also induces resistance to antineoplastic agents (13).

It has been widely demonstrated that cells expressing high c-Myc levels show an apoptosis-prone phenotype in response to different stimuli, affecting cell cycle phases (14).

Paclitaxel is cytotoxic to many types of cancer cells (15-17) and used as one of the most effective chemotherapeutic drugs for ovarian and adrenocortical cells (18,19). Paclitaxel treatment arrests the cell cycle at G₂-M phase by preventing the depolymerization of microtubules, resulting in apoptotic cell death (20-22).

In this study we investigated whether the c-Myc expression level could modulate the multiple effects of paclitaxel in H295R and SW13 ACC cell lines, which are characterized by different c-Myc expression levels.

Our data indicate that a high c-Myc expression level (endogenous SW13 and overexpressing induced by transfection in H295R cell lines, respectively) potentiates the paclitaxel

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apoptogenic effects suggesting a relationship between c-Myc expression and responsiveness to paclitaxel. This finding indicates a hypothetical role of c-Myc as useful prognostic factor in patients with advanced ACC.

Materials and methods

Cell culture and treatment. Our study was conducted on H295R and SW13 cell models, obtained from American Type Culture Collection (ATCC; LGC Standards Srl, Sesto San Giovanni, Milan, Italy). H295R cells were obtained from a functional human ACC which has the ability to produce steroid hormones. On the contrary, the SW13 cell line derives from a small cell carcinoma in the adrenal cortex. These cells are not able to produce steroids and their exact histopathologic origin has not been completely elucidated (23). Both human ACC H295R and SW13 cells were maintained in a 37°C incubator in an atmosphere of 5% CO₂ in humidified air. H295R cells were grown in a culture medium consisting of a mixture of 1:1 DMEM/F12, enriched with insulin/transferrin/selenium, 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. SW13 cells were cultured in Leibovitz's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Both cell lines were treated with paclitaxel (EBEWE Pharma) at a final concentration of 100 and 1 nM, respectively.

Trypan blue analysis. Cell number was determined using a hemocytometer and the viability was assessed by their ability to exclude trypan blue. After trypsinization, cells were mixed with equal volume of 0.4% trypan blue in PBS and the percentage of stained cells was determined.

Cell cycle analysis and apoptosis. The cell cycle was evaluated by using propidium iodide (PI; ICN Biomedicals Inc., Irvine, CA, USA) staining and flow cytometry (FCM) analysis. Samples were then measured by using a FACScan cytofluorimeter (Becton-Dickinson, Franklin Lakes, NJ, USA). Twenty thousand events per sample were acquired. The cell cycle phase percentages were estimated on linear DNA histograms by applying MODFIT software.

Apoptosis induction was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Mannheim, Germany) by using FCM. Briefly, trypsinised adherent cells and floating cells were pooled, washed once with PBS (Lonza, Verviers, Belgium) and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Samples were then permeabilised in 0.1% Triton X-100 (Sigma-Aldrich) in 0.1% sodium citrate (Sigma-Aldrich) and washed with PBS (Lonza). Each sample was incubated in 50 µl reaction mixture [terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP] for 1 h at 37°C, washed in PBS (Lonza) and then measured by FCM at 24, 48 and 72 h.

Western blot analysis. Cellular lysates were sonicated on ice, clarified by centrifugation at 20,000 x g and stored at -80°C. An aliquot of the cell lysates was used to evaluate the protein content by colorimetric assay. Total protein content (50 µg) were electrophoresed on 8% polyacrylamide gel in

the presence of SDS and transferred onto a nitrocellulose membrane. Blots were blocked for 1 h at room temperature with 5% non-fat dry milk in T-PBS buffer. Treated and untreated cells were incubated with the anti c-Myc 1:200 (Santa Cruz Biotechnology, CA, USA) cyclin B1 1:200 (Santa Cruz Biotechnology), pro-caspase 1:200 (Santa Cruz Biotechnology), and β-actin 1:10,000 (Sigma-Aldrich). The visualization of the antigens was done by enhanced chemiluminescent detection reagents by ECL. The analysis of bands was performed with Image J (Image Processing and analysis in Java) software program.

Transient transfection by siRNAs and pcDNA3-hMYC. Transfection of H295R cells was performed with the pINDneo (empty vector) plasmid and 3 µg pcDNA3-hMYC plasmid expressing the full-length C-MYC coding sequence driven by the CMV promoter for achieved C-MYC overexpressing cell clones. The SW13 cells were transfected with siRNA oligonucleotides for C-MYC. Control cells were transfected with a negative control siRNAgenome 200 pmol by non-targeting siRNA C-MYC (Dharmacon, Lafayette, CO, USA). Transfection was performed by incubating H295R cell line with 3 µg of pcDNA3-hMYC and pINDneo and SW13 cell line with 200 pmol of siRNAgenome and siRNA C-MYC using in both 10 µl of FuGene HD (Roche) according to the manufacturer's protocol. After overnight incubation, cells were maintained in fresh growing medium for 24 h and then were exposed to Taxol treatment at 100 and 1 nM concentration for H295R and SW13 respectively. Overexpression and downregulation of c-Myc protein levels on both cell lines were detected using western blot analysis.

Statistical analysis. All experiments were repeated at least three times and each experiment was carried out at least in duplicates. To determine global mean differences all samples were evaluated using the analysis of variance (ANOVA) and Student's t-test. A P-value <0.05 was considered statistically significant.

Results

Paclitaxel inhibitory effects on adrenocortical cancer cell growth. A dose-response curve (1, 10 and 100 nM) was used to compare the chemotherapeutic paclitaxel effects on both H295R and SW13 ACC cells. Fig. 1 clearly shows that there is a different response in the analysed cell lines as indicated in Fig. 1A; the IC₅₀ value of paclitaxel was 1 nM for the SW13 cells, whereas in the H295R cells this concentration was not able to induce any alteration in cell growth. In both treated and untreated H295R cells there is the same trend (Fig. 1B). It was necessary to increase the drug concentration to 100 nM to obtain a significant inhibition of cell growth in this cell line. In particular, just after 24 h of paclitaxel exposure cell growth inhibition was observed (43% using 1 nM in SW13 and 20% using 100 nM in H295R vs control, respectively) in both cell lines as shown in Fig. 1A and B. Incubating the cells with paclitaxel up to 72 h, the inhibitory effects reached ~66 and 58% in SW13 and H295R cells, respectively, at the doses 1 nM for SW13 and 100 nM for H295R cells (Fig. 1A and B).

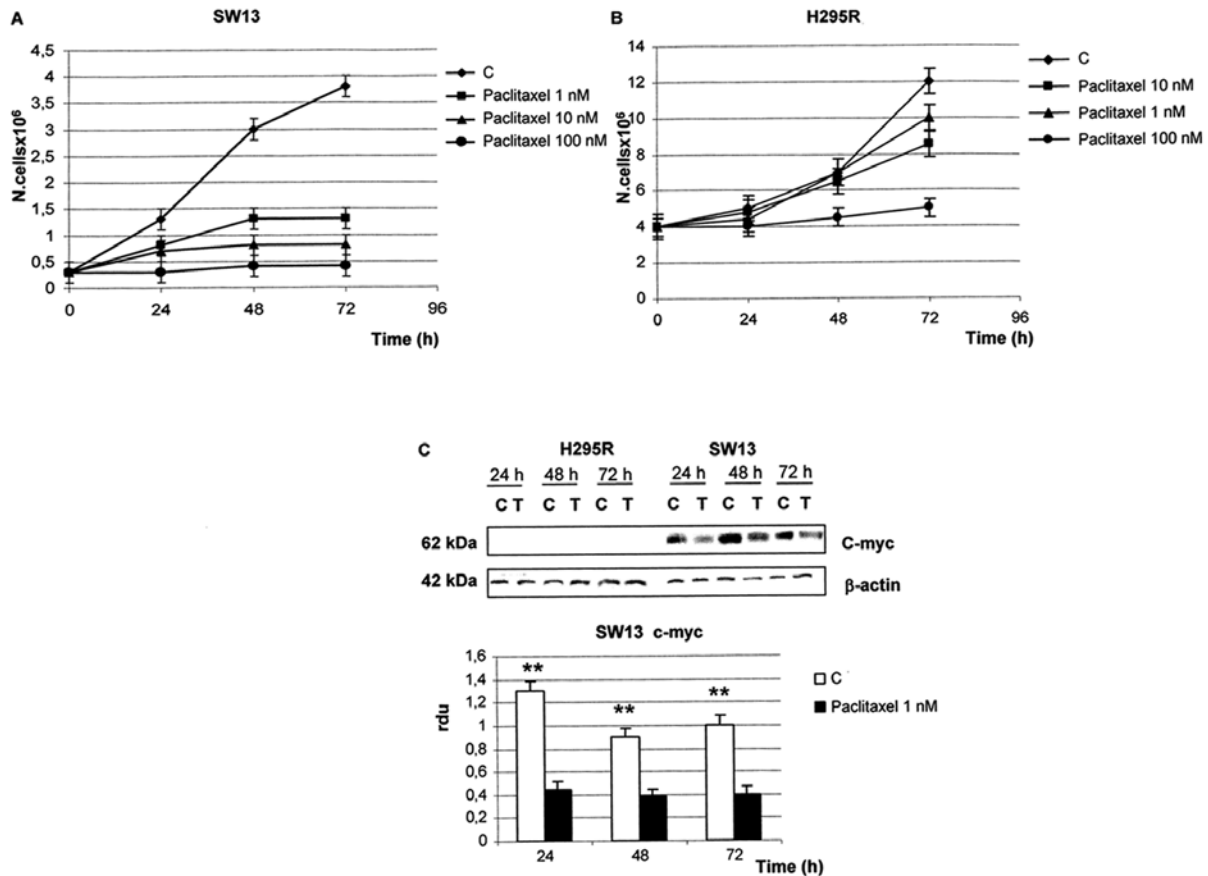


Figure 1. Paclitaxel treatment inhibits cell growth proliferation in ACC cell lines. (A) SW13 and (B) H295R cell lines were treated for 72 h with paclitaxel at different concentrations (1-100 nM) and the cell number was counted daily. (C) Expression levels of c-Myc protein in H295R and SW13 cell lines by western blotting. The cells were treated with 100 and 1 nM of paclitaxel, respectively. H295R cell line shows no signal for c-Myc either in control or in treated samples. On the contrary, in the SW13 cells a modulation of the c-Myc level was evident, with a significantly lower expression in treated samples than controls after 48 and 72 h. The bar graph represents the quantified mean \pm SD protein value relative to untreated cells, taken from three independent experiment. β -actin was used for normalization (** $P < 0.01$).

Cell viability in both cell lines was not affected by treatment as confirmed by using the trypan blue dye exclusion test (data not shown). Exposing SW13 cell line to 100 nM of paclitaxel a growth inhibition was observed due to a toxic effect of the chemotherapeutic on cell viability, as evidenced by the trypan blue test (data not shown). Since paclitaxel blocks cell division and since it has been proposed that expression of the C-MYC oncogene plays a pivotal role in the regulation of mammalian cell growth and its expression is associated with aggressive and poorly differentiated status (24), c-Myc protein expression level was analysed in both cell lines by western blot analysis.

As shown in Fig. 1C, we observed that in the SW13 cells the c-Myc endogenous expression was already affected by paclitaxel at 24 h after treatment. In fact, 70% of c-Myc protein was downregulated. Moreover, this trend was maintained until 72 h after treatment in treated compared to untreated samples ($P < 0.05$). In contrast, the endogenous protein levels were undetectable in both treated or untreated H295R cells, as shown in Fig. 1C ($P < 0.05$).

Considering the data, we concluded that the SW13 cells were more sensitive to paclitaxel exposure than the H295R cells. In SW13 cells 1 nM of paclitaxel was used to induce the biological effect, to obtain similar effects on H295R

cells, the paclitaxel dose was increased to 100 nM. The data evidenced that different response to paclitaxel exposure might be related to the expression levels of c-Myc in the two ACC cell lines.

Paclitaxel and cell cycle regulation. Many studies show that paclitaxel treatment causes arrest at G_2 phase of cell cycle (20-25). In this study, we evaluated the effects of paclitaxel in cell cycle distribution in treated and in control cells by flow cytometry analysis.

We stressed the analysis of paclitaxel exposure with 1 nM on H295R cells to point out the differences between the two ACC cell lines in response to paclitaxel at the same dose. As shown in Fig. 2A, paclitaxel treatment at 1 nM dose did not interfere with cell cycle distribution in the H295R cells at any time-points (24, 48 and 72 h). There was no change in the cell cycle phases compared to control cells in any of the time periods considered.

To further investigate the absence of any effects of paclitaxel at 1 nM exposure in H295R, analysis of apoptosis by cytometry using TUNEL assay was performed. The results obtained evidenced no change in cell viability in fact as shown in Fig. 2B, there was not significant difference in TUNEL positive cells between treated and untreated cells. Thus, the

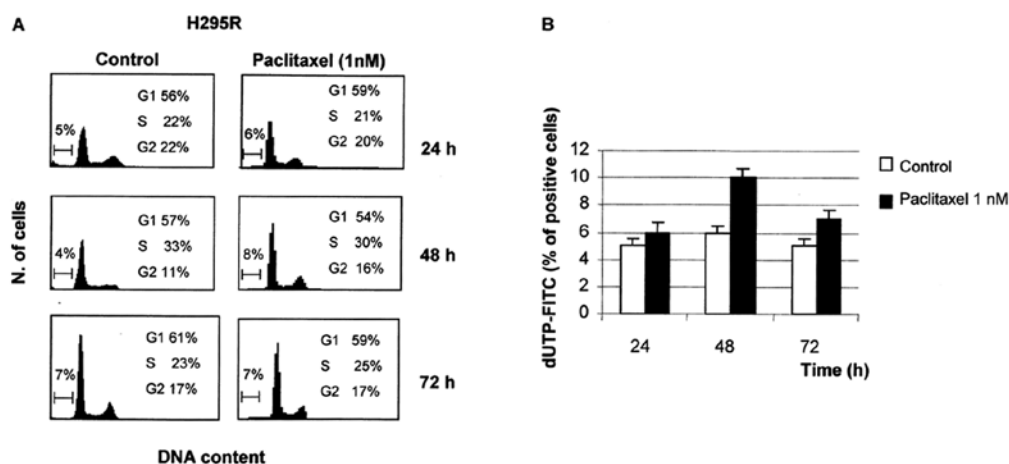


Figure 2. Effect of paclitaxel exposure (1 nM) on the cell cycle in H295R cells. Cell cycle analysis in the H295R cells after treatment. (A) Flow cytometric cell cycle analysis evaluated by PI staining and DNA content profiles in the paclitaxel-treated and the untreated H295R cells at a concentration of 1 nM. This treatment does not interfere with cell cycle progression at any time-points (24-72 h). (B) Detection of apoptosis by TUNEL assay at 24-48-72 h. Flow cytometric analysis does not show a significant difference in treated and untreated samples. It clarifies definitively that paclitaxel treatment at a dose of 1 nM in H295R cells did not induce any modification either on cell cycle distribution or on cell death. Results are representative of at least three independent experiments.

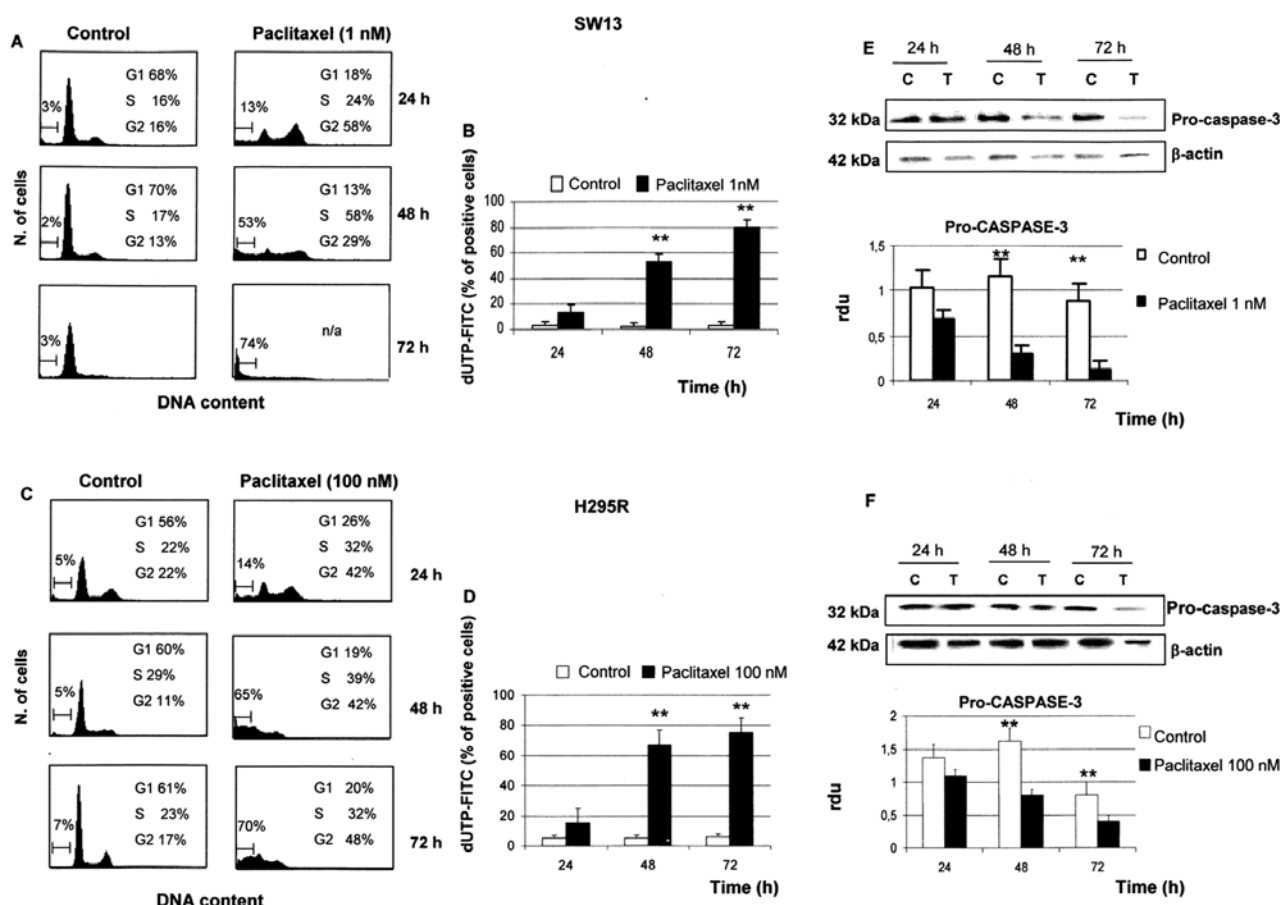


Figure 3. Effects of paclitaxel exposure at different concentrations on cellular proliferation in SW13 and H295R cells. Flow cytometric cell cycle analysis was evaluated by PI staining and DNA content profiles in SW13 (A) and H295R (C) cells untreated and paclitaxel-treated at 1 and 100 nM, respectively. In both cell lines a cell accumulation was evident at the G₂ phase of cell cycle at 24 h after treatment (58% in SW-13 paclitaxel treated compared to 16% in untreated control samples; 42% in H295R treated compared to 22% in untreated cells). Paclitaxel treatment induced gradual apoptotic cell death in both cell lines, as shown by the percentages of sub-G₁ region (74% in SW13 and 70% in H295R cell lines) at 72 h after treatment. Detection of apoptosis by TUNEL assay was evaluated at 24-72 h after treatment in SW13 (B) and in H295R (D). Data reported represent the percentage of treated cells with Taxol compared with untreated cells, and are the average of at least three separate experiments. Western blot analysis of caspase-3 in the SW13 (E) and in H295R cells (F) 24-72 h after exposure to paclitaxel and related controls. Each lane was loaded with 50 μ g of protein from cell lysates. The relative amount of transferred protein was quantified by estimating the relative density units normalized to β -actin content. Pro-caspase-3 levels, in treated cells, were significantly reduced particularly in SW13 (75% vs control) compared to H295R (50% vs control) cell lines at 72 h after treatment. Bar graph represents the quantified mean \pm SD protein value relative to untreated cells. β -actin was used for normalization (**P<0.01). Reported results are representative of at least three independent experiments.

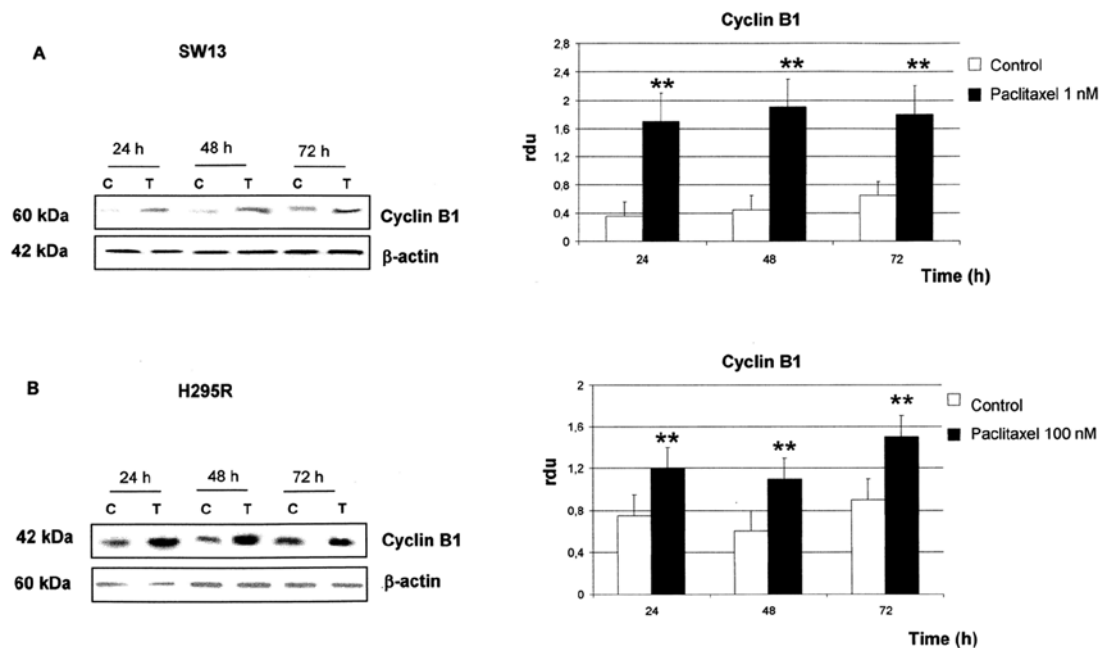


Figure 4. Upregulation of cyclin B1 by paclitaxel exposure in ACC cell lines. (A) Western blot analysis of cyclin B1 in the SW13 cell line after treatment. Paclitaxel at 1 nM induced an increase of cyclin B1 signal from 24 h up to 72 h. Each lane was loaded with 50 μ g of protein from cell lysates. The relative amount of transferred protein was quantified by estimating the relative density units normalized to β -actin content. (B) Representative western blot experiment of cyclin B1 in the H295R cells after Taxol exposure. Each lane was loaded with 50 μ g of protein from cell lysates. Cells treated with 100 nM of the drug showed a higher expression level of cyclin B1 at all experimental time-points. The relative amount of transferred protein was quantified by estimating the relative density units normalized to β -actin content (** $P < 0.01$). Images are representative of at least three independent experiments. The respective bar graphs are shown.

paclitaxel treatment at dose of 1 nM in the H295R cells did not induce any modification on cell growth, such as on cell cycle distribution or on cell death (Fig. 2).

In order to compare the effect of two different paclitaxel concentrations (1 and 100 nM) in SW13 and in H295R cells, respectively cell cycle analysis using flow cytometry was performed (Fig. 3). As shown in Fig. 3A in the SW13 cells, paclitaxel 1 nM led to an increase of the cell population in the G_2 phase of the cell cycle at 24 h (58%), compared to control (16%). In the H295R cells the paclitaxel effects (100 nM) were quite similar to those observed in SW13 treated with 1 nM of paclitaxel, in fact G_2/M cell cycle perturbation was evident already after 24 h of treatment (42% in treated vs 22% in control cells) (Fig. 3C). In addition the different concentrations (1 nM in SW13 and 100 nM in H295R cells) of the paclitaxel were able to induce apoptosis in both cell lines. As indicated in Fig. 3A and C, the incidence of apoptosis measured as the cells in the sub- G_1 region of DNA histogram demonstrated that paclitaxel treatment induced cell death. The percentage of apoptosis was 74% in SW13 vs 70% in H295R cells after 72 h. TUNEL assay was used to confirm the apoptotic cell death: Fig. 3B-D reveal the occurrence of apoptosis in both cell lines after paclitaxel exposure.

We further investigated whether apoptosis occur through regulation of expression of pro-caspase-3 by using western blot analysis. As indicated in Fig. 3E and F the level of pro-caspase-3 protein decreased dramatically during apoptosis, particularly in the SW13 cells compared to H295R cells after 72 h. Pro-caspase-3 level decreased in a time-dependent manner in the SW13 cells, reaching ~75% inhibition at 72 h

from treatment when compared to control. Similar result was obtained in H295R cells, in which paclitaxel treatment affected pro-caspase-3 level after 24, 48 and 72 h when compared to untreated cells as shown in Fig. 3F. Endogenous levels of pro-caspase were inhibited of 50% and this trend was maintained until 72 h after paclitaxel exposure ($P < 0.05$). All the data confirmed that paclitaxel affects ACC cell growth via apoptosis induction.

Paclitaxel modulates the expression of cell cycle regulators on ACC cell lines. We next performed western blot analysis to examine the protein expression level of G_2/M phase such as cyclin B1.

In both SW13 and H295R cells, cyclin B1 protein expression remained up-regulated from 24 until 72 h after paclitaxel treatment. As shown in Fig. 4A, the cyclin B1 level in the SW13 cells was already upregulated (81%) at 24 h and this trend was maintained for 48 and 72 h after paclitaxel exposure at 1 nM, when compared with untreated control ($P < 0.05$). Similar results were found in H295R cells. As shown in Fig. 4B, the paclitaxel exposure resulted in strong upregulation of cyclin B1 level between 24 and 72 h post-treatment (42, 45 and 40% vs control cells at 24, 48 and 72 h) ($P < 0.05$).

C-MYC in paclitaxel cellular responses in adrenocortical cancer cells. To further evaluate the contribution of C-MYC to treatment responses and its biological consequences, both H295R and SW13 cell lines were transiently transfected with pcDNA-hMYC and siRNA for overexpression and downregulation of the c-Myc protein, respectively.

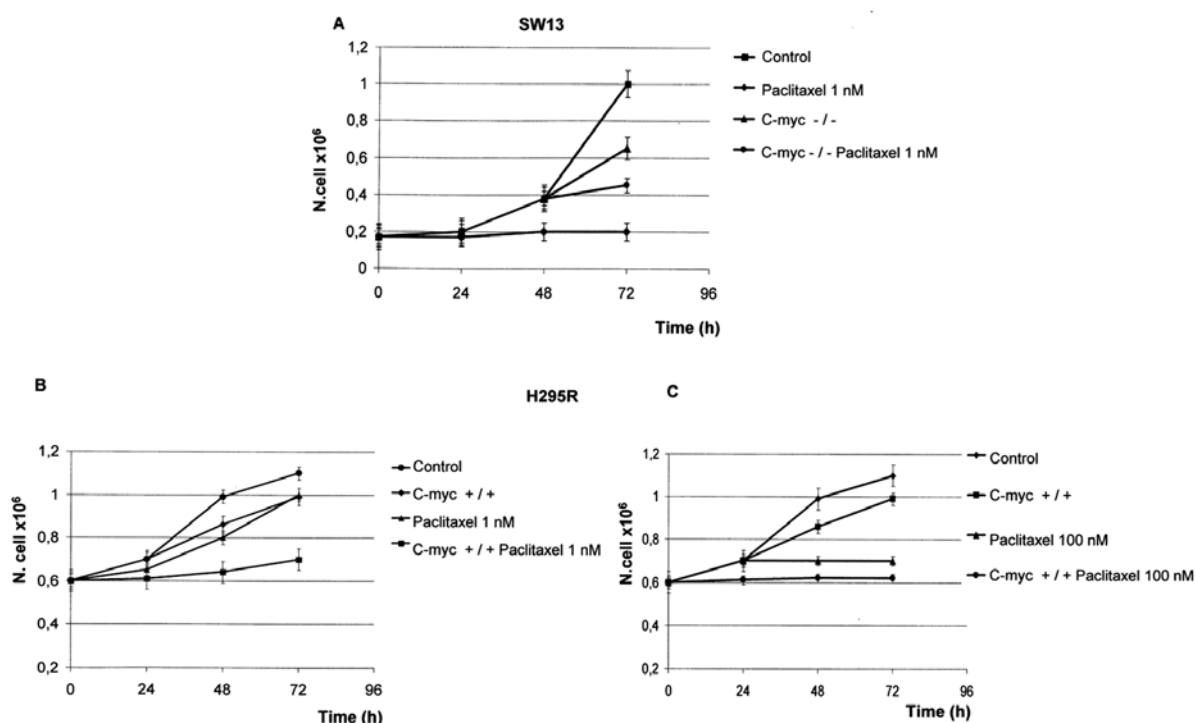


Figure 5. Effect of c-Myc protein on ACC cell proliferation after paclitaxel exposure. (A) Growth curve of SW13 cells: untreated used as control, paclitaxel-treated, untreated and silenced for Myc protein (C-MYC^{-/-}), paclitaxel-treated and silenced for Myc (C-MYC^{-/-} paclitaxel 1 nM). This experiment showed that by silencing c-myc, the reduction of cell growth, induced by paclitaxel, was less effective compared to cells with endogenous C-MYC paclitaxel-treatment ($P < 0.05$). (B) Growth curve of H295R cells: untreated for control, untreated and overexpressing c-Myc protein (C-MYC^{+/+}), paclitaxel treated, treated and overexpressing (C-MYC^{+/+} paclitaxel 1 nM). In H295R the overexpression of C-MYC rendered these cells more susceptible to the paclitaxel effect ($P < 0.05$). (C) The growth curve of the H295R cell line treated with 100 nM paclitaxel.

Western blot analysis results showed that the expression of c-Myc in H295R and SW13 cells after transfection was higher and lower, respectively, than in the untransfected cells (data not shown).

The reduction of endogenous c-Myc protein level by transient transfection by C-MYC siRNA in SW13 did not affect significantly cell growth as shown in Fig. 5A. This result was similar when SW13 cells were treated with paclitaxel 1 nM after siRNA transfection for C-MYC, in fact only 30% inhibition of cell growth was observed when compared to cells transiently transfected (Fig. 5A) ($P < 0.05$).

The susceptibility of SW13 cells to paclitaxel (1 nM) treatment was confirmed only in samples with higher c-Myc endogenous level (80% of inhibition when compared to control cells) (Fig. 5A) ($P < 0.01$), suggesting a role of c-Myc protein in mediating the cell growth inhibition after paclitaxel exposure.

The c-Myc overexpression in the H295R cells by transient transfection render the cells more susceptible to paclitaxel effect. As shown in Fig. 5B, the treatment with 1 nM of paclitaxel in cells with C-MYC overexpression, induced an inhibition of 20 and 30% when compared to untreated cells after 48 and 72 h, respectively. While the presence of the C-MYC, overexpressed by transient transfection, did not interfere with cell growth; the same trend was observed in both paclitaxel untransfected cells and control (untreated and untransfected cells) (Fig. 5B).

The effects of paclitaxel at 100 nM concentration were not different in untransfected and transfected samples overexpressing c-Myc protein as evidenced in Fig. 5C.

The data are consistent with previous results obtained from SW13 cells, in which the presence of the c-Myc protein is important for rendering cells more susceptible to chemotherapy treatment. These results suggest that c-Myc plays a key role in the regulation of cell growth after paclitaxel exposure.

We also investigated the effect on the cell growth curve by cell cycle progression analysis using flow cytometry after transient transfection of both ACC cell lines. The experiments were conducted at 48 h, time in which we previously (Fig. 3), observed a significant perturbation of the cell cycle and an induction of apoptotic cell death.

Flow cytometry analysis indicated that siRNA/paclitaxel treatment caused a cell cycle arrest at G₂ phase (Fig. 6A), confirming previous data concerning the involvement of c-Myc in the response to paclitaxel treatment. This effect is accompanied by apoptosis induction as evidenced by TUNEL assay (Fig. 6B). Moreover, no difference in cell cycle distribution or signs of apoptotic cell death were observed in the other samples (Fig. 6A and B).

The above is consistent with the dependence of c-Myc and chemo-responsiveness. Furthermore, H295R cell line analysis revealed that the c-Myc overexpression using a pcDNAhMYC induced a strong perturbation of cell cycle (Fig. 6C) characterized by apoptotic cell death (Fig. 6D).

The data on pcDNA-hMYC (C-MYC cells overexpressing), pINDneo/paclitaxel and pINDneo (cells transfected and paclitaxel 1 nM and cells transfected with empty vector, respectively), evidenced neither a significant cell cycle perturbation nor apoptosis-mediated cell death.

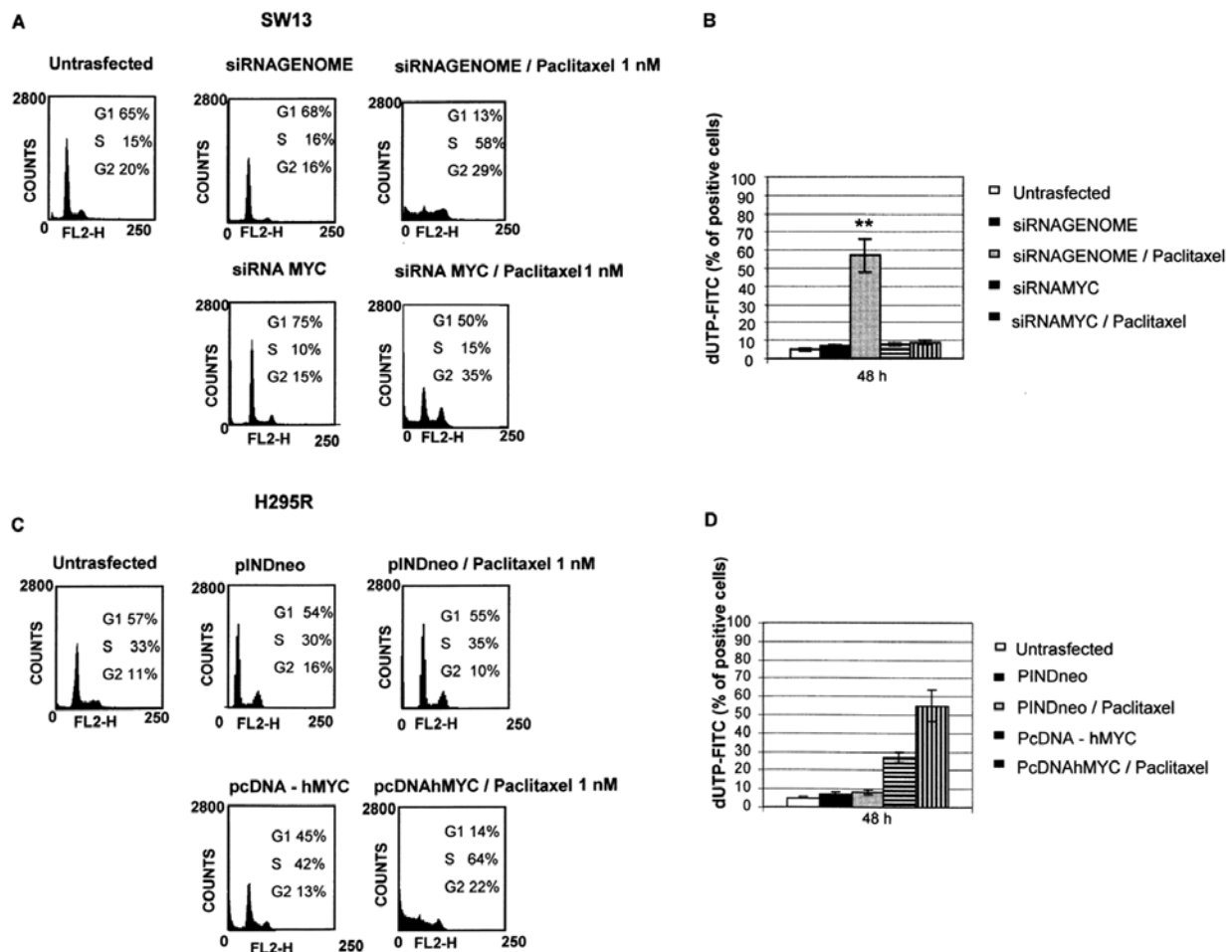


Figure 6. Effect of paclitaxel in adrenocortical cancer cells under- and overexpressing C-MYC. (A) Flow cytometric analysis of SW13 cell line untransfected and transfected with siRNAGENOME (empty vector), transfected with siRNAGENOME and treated with paclitaxel 1 nM, untreated and transfected with siRNA MYC and transfected with siRNA MYC treated with paclitaxel 1 nM. Cells were stained with propidium iodide and then analyzed for DNA content. DNA content cell percentage is indicated in the relative boxes. Paclitaxel treatment, in SW13 sample cells transfected with siRNAGENOME, promotes an accumulation at the G₂ phase followed by apoptotic cell death. On the contrary, SW13 C-MYC silenced cells showed neither cell cycle perturbation nor apoptosis. (C) Flow cytometry of the H295R cell line untreated, transfected with pINDneo (empty vector) and untreated, transfected with pINDneo paclitaxel-treated 1 nM, transfected with pcDNA-hMYC (overexpressing C-MYC) and untreated, transfected with pcDNA-hMYC paclitaxel-treated. In the H295R cell line C-MYC overexpression (pcDNAhMYC/paclitaxel) induced a significant perturbation of cell cycle with apoptotic cell death whereas no biological effect is observed in the other samples. The bar graphs (B and D) represent the detection of apoptosis evaluated by flow cytometry at 24 h after treatment in SW13 and H295R, respectively (**P<0.01). Images are representative of three replicates.

In conclusion, c-Myc enhances the cytotoxic effect of paclitaxel in ACC SW13 and H295R cell lines underlining its dependence on the treatment dose.

Discussion

ACC is a rare endocrine cancer with unfavorable prognosis. There is no evidence indicating a gold standard protocol for ACC treatment. The major problem is the lack of early diagnosis and the risk of metastatic spread (3). Metastatic ACC usually leads to death within 1-3 years. An obstacle is due to drug resistance to standard cytotoxic chemotherapy (26).

The mainstay of ACC therapy at the time of initial evaluation (27-29) is complete surgical removal of the adrenal mass whereas cytotoxic chemotherapy has been used extensively in advanced stages of the disease. However, response rates are often unsatisfactory (29-33). The use of the insecticide-derivative o,p'-DDD (mitotane), either as single agent or in

combination with other drugs, generally shows a response rate of 20-33% (31-33). Other therapies of ACC have been tested and FIRM-ACT study was completed recently. The results show that patients treated with etoposide, doxorubicin and cisplatin (EDP) in repeated cycles, in association with mitotane had a significantly higher response rate than those treated with streptozotocin-mitotane only. No difference was observed in overall survival confirming the poor prognosis in patients affected by advanced ACC (34).

C-MYC gene amplification, maintaining the transformed state, appears to underlie c-Myc overexpression in many cases of carcinoma and its overexpression is associated to disease stage and progression in other cancer types (11). Some authors have reported the importance of the c-Myc protein level since Szabó and colleagues (10) in a meta-analysis and review of gene expression microarray and comparative genome hybridisation (CGH) performed in a wide range of adrenal samples, observed downregulation of C-MYC in ACC tissue compared

to normal adrenal cortex and benign adrenocortical tumors. They justify these findings with the loss of chromosome 8q24 where the C-MYC is located suggesting that C-MYC downregulation represents a crucial event in adrenocortical tumorigenesis.

Many reports emphasize the role of C-MYC modulation in therapeutic strategy against cancer (35-37). Likewise, we demonstrated this in two previous reports, as the modulation of this oncogene can also sensitize cells towards apoptosis in response to cytotoxic insults including chemotherapeutic agents and radiotherapy (38,39).

Some cytotoxic compounds were identified, and currently used in oncology, able to reduce tumor cell viability and induce apoptosis in a C-MYC-dependent manner on a wide variety of cell lines (40). In the series of the drugs tested, paclitaxel, a microtubule target, revealed increased activity in cells overexpressing C-MYC. The authors advocated further studies elucidating the mechanisms underlying their specificity towards C-MYC.

Paclitaxel has experimental and clinical efficacy on many malignant tumors, thus allowing a wide spectrum therapeutic success (41). The antineoplastic mechanism of paclitaxel is to block the cell cycle of cancer cells, leading to apoptosis (20,21). A previous (19), and a recent report (42) described a positive effect of paclitaxel in ACC cell lines and in mouse xenograft ACC models, indicating a potential clinical use of the drug.

On the basis of the antineoplastic properties of paclitaxel and for the need to study C-MYC expression in ACC cell lines, as advocated by Szabó and colleagues (43) in the present study, we investigated the c-Myc expression level in H295R and SW13 cell lines and its role in response to paclitaxel chemotherapy. Our results showed a different expression pattern of c-Myc with detectable and undetectable level of this protein in SW13 and H295R cell lines, respectively. This result, in H295R cell line, confirming the previous data obtained by microarray analysis in ACC samples (7) allow to speculate that some defect that prevents protein translation occurs. The highest level of expression of c-Myc detected in SW13 is apparently inconsistent with the lower expression of C-MYC reported in the ACC. A possible explanation for this result may be a dependency on the origin of this cell line. Wang and Rainey (23) in a review on human ACC cell lines claim that SW13 cell line derived from a small cell carcinoma in the adrenal cortex and warn against the usefulness of these cells as an ACC model study. However, the increased expression of c-Myc in the SW13 and the lack in H295R cell lines is an intriguing model to study the role of c-Myc in relationship to chemotherapy. For this purpose we used the cell lines expressing different levels of the c-Myc protein to assess the paclitaxel effect in ACC *in vitro*.

Interestingly, two different concentrations of paclitaxel were necessary to contrast cell growth and induce apoptosis in ACC cell lines with different levels of c-Myc. In particular, a significant cell cycle perturbation, characterized before by an arrest at the G₂ phase and followed by apoptotic cell death, was observed with 1 nM paclitaxel concentration in the SW13 cell line. On the contrary, a paclitaxel concentration of 100 nM was necessary in the H295R cell line, where the endogenous c-Myc protein level was undetectable, in order to obtain the same antiproliferative effect observed with a lower chemotherapeutic concentration used in the SW13 cells. This

paradoxical difference prompted us to consider an effective role of c-Myc regarding paclitaxel treatment. The result of this study revealed two cell lines including one overexpressed (SW13) and the other with undetectable c-Myc (H295R). Intriguingly, we observed that the greater the c-Myc expression level (SW13) the lower the dose of the drug required. Whereas, in cell samples (H295R) not expressing c-Myc it was necessary to increase the paclitaxel dose.

In order to establish if there was a cause-effect relationship between paclitaxel treatment and c-Myc expression levels in ACC cell lines we have overexpressed and downregulated C-MYC in H295R and SW13 cells, respectively. Surprisingly, the transient transfection with pcDNA-hMYC and siRNA for overexpression and downregulation of the c-Myc protein in H295R and SW13 cells, respectively, was unable to reversed the antiproliferative effects observed in untransfected cell lines. Paclitaxel induced a lower growth inhibition in C-MYC silenced SW13 samples than in the control. Alternatively, the overexpression of c-Myc permitted to paclitaxel to inhibit cell growth leading to apoptotic cell death in H295R cells. It is striking to note that this effect was obtained at a paclitaxel concentration of 1 nM.

Upregulated or downregulated c-Myc level did not affect significantly cell growth in the cell lines. Our data are in agreement with Liu and colleagues (44) who showed that the overexpression of C-MYC by transient transfection assay was not correlated with proliferation in H295R cell line. This finding shows how other factors involved in the C-MYC pathway are essential for the occurrence to proliferative effect in cancer. Often, not only c-Myc expression level but also members of Myc family, including N-Myc and L-Myc, are found amplified and/or rearranged in tumors (45,46). This behavior suggests a role of c-Myc in mediating cell growth inhibition and cell death after paclitaxel exposure. Our data is in line with the observations by Horwitz (41) and Goga and colleagues (47) who suggested that C-MYC overexpressing cells are particularly sensitive to chemotherapeutic responses. Taken together these findings suggest that the downregulation of C-MYC gene and the lack of protein level in ACC could represent a hypothetical factor of chemoresistance. This hypothesis would justify the therapeutic failure occurring in ACC. Furthermore, considering the SW13 cell line as arising from metastasis to the adrenal cortex, as suggested by Leibovitz and colleagues (48) the presence of c-Myc could be considered as a prognostic marker of therapy.

In this study we have confirmed the effectiveness of paclitaxel as antineoplastic drug in the ACC cell lines. Moreover, we demonstrated a direct correlation between the drug concentration and the c-Myc expression levels, establishing that lower concentrations of the drug are necessary to induce an antiproliferative effect if expression level of c-Myc is greater, on the contrary, the drug dose must be greater in the case in which c-Myc is deficient or downregulated in ACC cell lines. This study highlights that C-MYC overexpression, either basal in SW13 or induced in H295R cells, was associated to a good response to paclitaxel chemotherapy, consisting in an evident perturbation of cell cycle followed by apoptotic cell death. Furthermore, this study, identifying c-Myc as a marker predicting paclitaxel response in ACC cell lines, could allow the use also in clinical setting. Thus,

the use of paclitaxel in chemotherapy strategies against ACC should be rationally based on the c-Myc molecular profile in this neoplasia. Our results achieved on H295R cells, considered a canonical ACC cell line, and on those found in the literature, do not suggest paclitaxel use in the primary ACC treatment. On the contrary, we believe that paclitaxel may be indicated in the treatment of metastatic ACC. Alternatively, it could be considered in the treatment of adrenal metastatic spread by other primary cancer. However, considering the ACC heterogeneity, it could be useful to assess the c-Myc expression level before starting paclitaxel treatment, in a clinical setting. Predicting response to chemotherapy would allow individualize therapy in ACC patients, thus increasing the therapeutic success probability. Although these findings are intriguing, they need further validations.

The evident lack of C-MYC showed in the present and in previous studies, on the representative H295R ACC cells and on microarray profile of ACC tissue, respectively, is particularly attractive for understanding the mechanisms underlying ACC tumorigenesis. We believe that the absence of a critical oncological pathway represents a crucial reasoning point in ACC pathogenesis.

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