Original article

Kv10.1 and p53 expression in SiHa cells during the generation of cisplatin resistance

Expresión de Kv10.1 y p53 en células SiHa durante la generación de resistencia a cisplatino

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ABSTRACT

Introduction: Cervical cancer is one of the pathologies with the highest incidence in women. Its conventional treatment is cisplatin-based combination chemotherapy. Since drug resistance is a limiting factor for treatment success, it is necessary to find biomarkers that allow us to show the development of chemoresistance, such as the Kv10.1 and p53 proteins, which are important in cisplatin-resistant tumor models.

Objective: This study aims to evaluate the variation in Kv10.1 and p53 protein expression in the SiHa cell line during the process of generating cisplatin resistance.

Methods: SiHa cells were cultured while exposed to different doses of cisplatin. Then, total protein extraction was performed and the changes in expression were evaluated via dot blot assays.

Results: A significant decrease was found in the expression of the Kv10.1 and p53 proteins at doses of 0.05 µg/mL and 0.5 µg/mL of cisplatin, with morphological changes associated with each dose.

Conclusions: The response mechanism to cisplatin exposure in the SiHa cell line differs from that reported for other types of cancer. There was no evidence of an increase in Kv10.1 expression; in contrast, its expression was similar to that observed for the p53 protein. Additionally, Kv10.1 and p53 did not present antagonistic behavior, so the resistance mechanism generated by cisplatin exposure is unrelated to the activity of both proteins.

Keywords: biomarkers; cisplatin; potassium channel kv10.1; SiHa cell line; p53 protein; drug resistance.

RESUMEN

Introducción: El cáncer de cuello uterino es una de las enfermedades de mayor incidencia en mujeres cuyo tratamiento convencional es la quimioterapia combinada basada en cisplatino. Siendo la resistencia a fármacos un factor limitante para el éxito del tratamiento, lo cual hace necesario encontrar biomarcadores que permitan evidenciar el desarrollo de la quimioresistencia, como las proteínas Kv10.1 y p53, de importancia en modelos tumorales resistentes a cisplatino.

Objetivo: Evaluar la variación en la expresión de las proteínas Kv10.1 y p53 en la línea celular SiHa, durante el proceso de generación de resistencia al cisplatino.

Métodos: Las células SiHa se cultivaron mientras eran expuestas a diferentes dosis de cisplatino y luego, se realizó la extracción de proteína total y se evaluaron los cambios en la expresión mediante ensayos de dot blot.

Resultados: Se encontró disminución significativa en la expresión de las proteínas Kv10.1 y p53 a las dosis de 0,05µg/mL y 0,5µg/mL de cisplatino, con cambios morfológicos asociados a cada dosis.

Conclusiones: El mecanismo de respuesta a la exposición de cisplatino en la línea celular SiHa difiere de lo reportado para otros tipos de cáncer y no se evidenció un incremento en la expresión de Kv10.1 y en contraste su expresión fue similar a la observada para la proteína p53. Adicionalmente, Kv10.1 y p53 no presentaron comportamientos antagónicos, por lo que el mecanismo de resistencia que se genera a la exposición a cisplatino es ajeno a la actividad de ambas proteínas.

Palabras clave: biomarcadores; cisplatino; canal de potasio kv10.1; línea celular SiHa; proteína p53; resistencia a fármacos.

Received: 01/12/2022

Aproved: 29/10/2023

Introduction

Cervical cancer is the most frequent cause of morbidity and mortality from gynecologic cancer and the fourth leading cause of death in women diagnosed with cancer. It accounts for approximately 604 000 new cases and 342 000 annual deaths worldwide.[1] Once the disease has been detected, the choice of treatment depends on the stage of the cancer, with advanced stages and recurrent and/or metastatic cancers requiring the administration of combinations of platinum compounds, such as cisplatin (cis-diamminedichloroplatinum II, CDDP) and carboplatin. Although many patients respond to this treatment, a significant percentage develop chemoresistant relapses that end up being life-threatening for the patient.[2]

Resistance to cisplatin and other chemotherapy drugs is directly related to the stage of tumor progression, given that the cancer cells develop additional genetic alterations that give them the advantage to grow as they proliferate. Consequently, the expected cytotoxic or cytostatic effect does not occur. Studies show that there is a differential expression of proteins between tumor cells that are resistant and sensitive to cisplatin, suggesting that proteins that are overexpressed in the resistant cells could be related to the development

of drug resistance. Those involved in ion transport, specifically ion channels, play an important role in regulating the concentration of cisplatin in cells, and, therefore, the resistance or sensitivity to treatment.[3]

Current research is looking for proteins with differential expression between cells that are sensitive and resistant to cisplatin treatment to clinically apply them as resistance biomarkers. This will allow identifying patients who are experiencing chemo resistant relapses, with the purpose of offering them adequate therapeutic management. Ion channels, notably potassium channels, control cellular homeostasis, membrane potential, and other signals that may be involved in tumor biology by promoting angiogenesis, invasiveness, metastatic spread, and other characteristics of cancer cells.[4] These channels play a significant role in the induction of apoptosis because they modulate the inflow and outflow of K^+ ions in the cell. Thus, variation in the expression of potassium channels and, therefore, in ion flow has been shown to limit the efficacy of proapoptotic chemotherapeutic agents.[5]

Kv10.1 and KCNH1 are the most studied potassium channels in cancer because of their relationship with the development of resistance to chemotherapy drugs.[6] Increased expression of potassium channels in any tissue is indicative of proliferation and malignant transformation,[4] suggesting that it could be a molecule of interest for diagnostic use in several types of cancer due to its potential as a biomarker.[7,8,9]

Kv10.1 expression is controlled by regulators of cell survival and proliferation, such as the p53 tumor suppressor protein and the E2F1 growth factor, which often present alterations in cancer.[10] The activity of protein p53 is targeted at DNA repair and at generating senescence and apoptosis to prevent inappropriate cell proliferation.[11] In addition, variation in its expression is directly related to resistance to different drugs, including cisplatin,[12] suggesting that p53 can also be used as a biomarker to determine tumor chemosensitivity.

One of the etiologic agents of precancerous lesions in cervical cancer is the presence of the human papillomavirus (HPV-16), which encodes two oncoproteins (E6 and E7) that act directly on the p53 and retinoblastoma (pRb) tumor suppressor genes. Upon binding to p53, E6 induces degradation via ubiquitination and additionally degrades the BAK pro-

apoptotic protein, resulting in resistance to apoptosis and increased chromosomal instability. On the other hand, the E7 oncoprotein degrades the activated pRb upon binding, and releases the E2F transcription factor. This promotes the expression of the E2F1 growth factor, which in turn influences Kv10.1 expression, initiating the cell division process as a result. Thus, the expression of viral oncogenes leads to cell immortalization in various different tissues, as well as an alteration in the expression of the Kv10.1 and p53 proteins.[8] This proves the significance of studying these two proteins during the process of developing cisplatin resistance in HPV-positive cell lines. Considering the relationship between the p53 protein and the Kv10.1 channel, this study aims to analyze the variation in the expression of Kv10.1 and p53 in the SiHa (HPV-16) cervical cancer cell line during the development of cisplatin resistance, so that these proteins can be considered as possible biomarkers of cisplatin resistance.

Methods

The HPV-16-positive SiHa cervical cancer cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and 1% streptomycin. The cells were incubated at 37°C with 5% CO_2. To confirm the identity of the SiHa cell lines, we used a Linear Array HPV genotyping test to detect 37 high and low risk viral genotypes, following the manufacturer's instructions (data not shown).

Obtaining resistant cells

To induce cisplatin (CDDP) resistance, the cell line was continuously exposed to progressive concentrations of cisplatin of 0.05 µg/mL, 0.1 µg/mL, 0.5 µg/mL, 1.0 µg/mL, and 2.0 µg/mL, as instructed by *Roy* and *Mukherjee et al.*[13]. Each exposure had a recovery phase and confluence phase (resistance stage), as reported by *Wen et al.*[14]. In summary, the cells were incubated with exposure to each concentration until reaching over 80% confluence. Once reached, the cells were trypsin zed with a subsequent increase in the dose of the chemotherapy drug. The cell line without exposure to the chemotherapy

drug was cultured in the supplemented DMEM and was kept during this period as the control line. We cryopreserved aliquots of each sample to generate a stock of the cell line in its different stages of cisplatin resistance.

Identification of the Kv10.1 and p53 proteins using dot blot

We extracted the total protein for each stage of resistance, which was quantified using the bicinchoninic acid method (BCA) with spectrophotometer readings at 562 nm. Protein detection was conducted using the dot blot technique, following the protocol used by *David Stott*.[15] We used the anti-Eag1 (donated by Dr. Walter Stümer and Dr. Luis Pardo, researchers at the Max Planck Institute) and anti-p53 (DO-7, Thermo Fisher Scientific) monoclonal antibodies. We used anti-GAPDH (Developmental Studies Hybridoma Bank, P4C10, E7-s, and DSHB-hGAPDH-2G7) as a loading control. Anti-mouse secondary antibodies (Anti-Mouse IgG) were used for the final detection.

Dot signal detection was achieved using chemiluminescence (Thermo Scientific™ Super Signal™ West Pico PLUS Chemiluminescent Substrate) via imaging with a Thermo Fisher Scientific photo documentation system (I Bright FL1000).

The intensity of each image was determined using the ImageJ software by selecting the dotted area in the program, thus obtaining an intensity value calculated by transforming the pixel values of the selected area into brightness values.

Statistical analysis

Each assay was conducted in triplicate. The data were analyzed using Rx 64 (Core Team, 2016) and are presented as the mean ± standard deviation. We performed normality and homoscedasticity tests, as well as parametric and non-parametric tests. A p-value of <0.05 was considered to indicate a statistically significant difference.

Results

Obtaining the chemo resistant SiHa cell line

We confirmed the HPV-16 serotype for the SiHa cell line (data not shown) as a preliminary step. Subsequently, cisplatin resistance was induced in the SiHa cell line using 5 concentrations of cisplatin: 0.05 µg/mL, 0.1 µg/mL, 0.5 µg/mL, 1.0 µg/mL, and 2.0 µg/mL. Generating these stages of resistance took 30 weeks, during which the cells presented morphological changes such as elongation, irregular membrane borders, and appearance of granules in the cytoplasm (fig. 1. B-G). This differed from cells without exposure to cisplatin, in which no cytoplasmic granules or membrane alterations were observed (fig. 1.A). However, these changes were progressive with the increasing concentrations of the chemotherapy drug. In addition, it was shown that higher doses required more time to obtain resistant phenotypes.

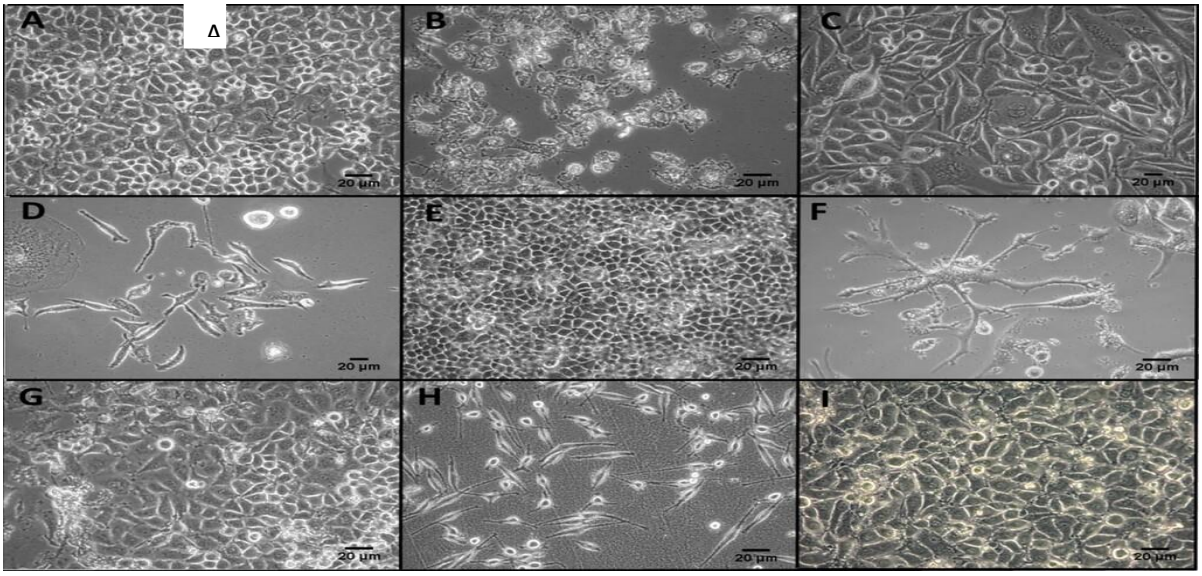

Fig.1 - Photographs of SiHa cells exposed to different concentrations of cisplatin during the resistance generation process **A**. Unstimulated SiHa cells, **B**. SiHa cells exposed to 0.05 µg/mL CDDP, **C**. SiHa cells resistant to 0.05 µg/mL CDDP, **D**. SiHa cells exposed to 0.1 µg/mL CDDP, **E.** SiHa cells resistant to 0.1 µg/mL CDDP, **F**. SiHa cells exposed to 0.5 µg/mL CDDP with evident morphological changes, **G**. SiHa cells resistant to 0.5 µg/mL CDDP, **H**. SiHa cells exposed to 1.0 µg/mL CDDP, with debris in the cell medium corresponding to dead cells due to the chemotherapy drug, **I**. SiHa cells resistant to 1.0 µg/mL CDDP

With the first cisplatin concentration, the cells did not present considerable morphological changes.

A cell mortality of approximately 20 % of the total confluence was registered in the first three days of exposure (fig.1B).

Cytoplasmic granules also became evident (fig. 1.C). Due to the rapid tolerance of this dose, the drug concentration was increased the following week. The cells remained for 30 days at the 0.1 µg/mL dose.

The first three days presented approximately 70% cell death (fig. 1.D) and the growth rate decreased considerably compared to unstimulated cells.

Morphological changes could be seen in the first 20 days and subsequently, the cells started recovering their initial morphology, similar to that of the control cells, but retained the cytoplasmic granules throughout the dose (fig.1.E).

The cells were exposed to the 0.5 µg/ mL for one month to generate the third stage of resistance. 20% of the cell population died in the first eight days of exposure, and in the following 12 days, the population decreased to a minimum confluence of 30 %.

At this stage, the morphological changes were more evident (fig. 1.F). However, the cells returned to their usual form, preserving the cytoplasmic granules as in the previous doses (fig. 1.G).

These changes are further shown in figure 2, where the control cells are compared to the cells exposed to 1.0 µg/mL during and after generating cisplatin resistance (fig. 2.B and C).

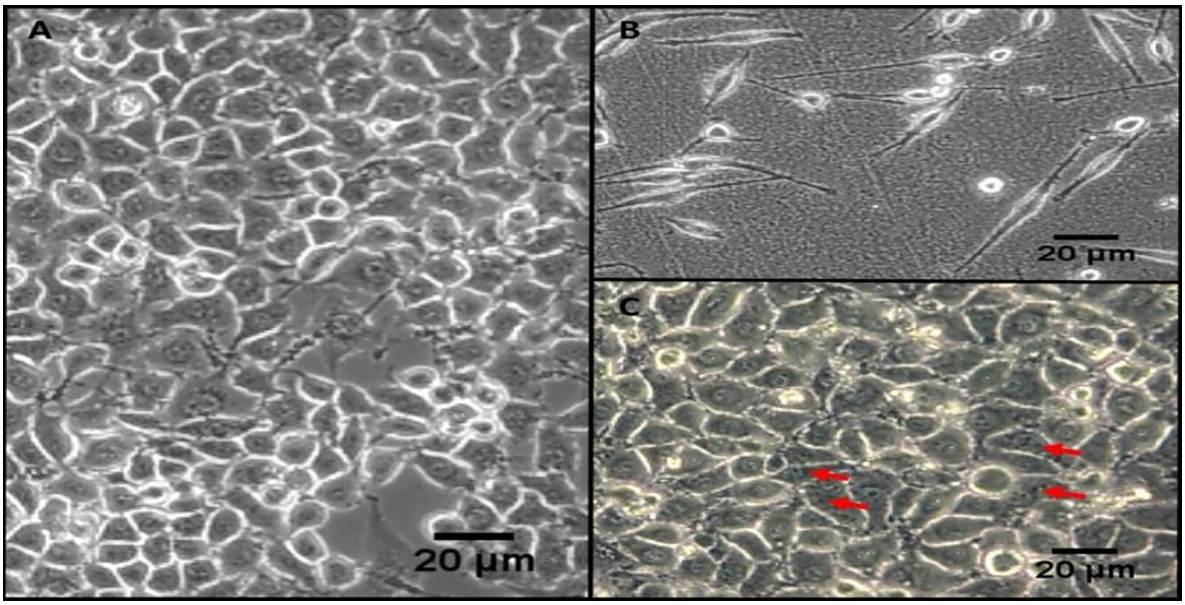

Fig. 2 - Comparison of SiHa cells resistant to 1.0 µg/mL cisplatin vs. unstimulated control cells. **A.** Unstimulated SiHa cells; **B.** SiHa cells exposed to 1.0 µg/mL CDDP; **C.** SiHa cells resistant to 1.0 µg/mL CDDP, where the presence of granules in the cytoplasm is evident (red arrows)

Dot blot of chemo resistant cells.

The dot blot analysis yielded significance values of $p < 0.05$ and $p < 0.001$ for Kv10.1 (fig. 3.A) and p53 (fig. 3.B), respectively. This indicates significant differences between protein expression and the cisplatin resistance stage.

However, despite the varying behavior of Kv10.1 and p53, it was not found that the increase in their expression had a proportional relationship to the increase in the concentration of the chemotherapy drug.

No significant differences were found for the protein used as the loading control, since GAPDH expression remained constant throughout the different stages of cisplatin resistance ($p = 0.5$).

This confirms that the variation observed in Kv10.1 and p53 expression is not due to a variation in the amount of protein seeded into each well, but rather that such variation is the result of changes in protein expression during the generation of cisplatin resistance (fig. 3.C).

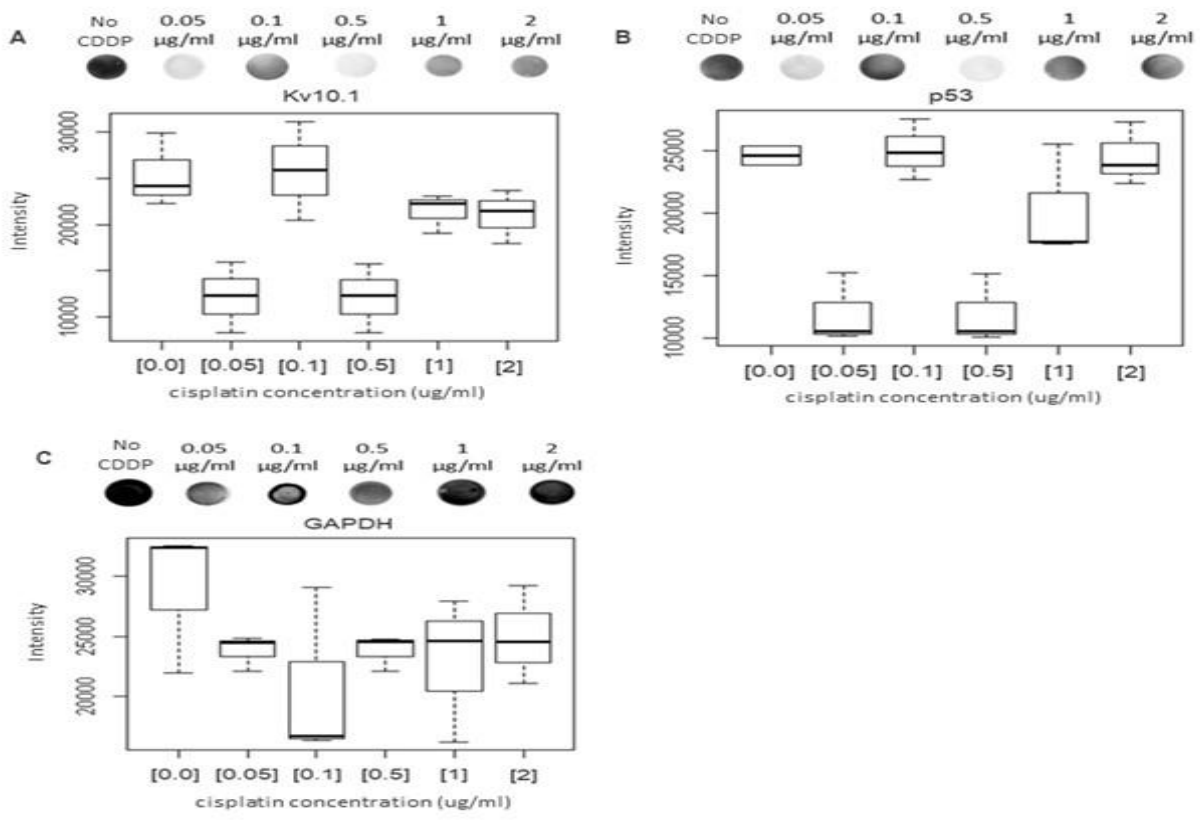

Fig. 3 - A. Intensity of Kv10.1 protein expression at different stages of cisplatin resistance (CDDP) (p=0.00162). **B.** Intensity of p53 protein expression at different stages of CDDP resistance (p=0.000317). **C.** Intensity of GAPDH protein expression at different stages of CDDP resistance

The behavior of the Kv10.1 and p53 proteins was variable at different stages of resistance. Drastic increases of cisplatin generated a significant decrease in the expression of both proteins (fig. 3A and 3B), such as with the first stimulus and then by increasing the dose fivefold (from 0.1 µg/mL to 0.5 µg/mL). In contrast, minor changes in drug doses (by doubling the doses) did not generate alterations in the expression of these proteins.

This shows that there is a statistically significant variation in the expression of these proteins when there is a high increase in the dose of cisplatin.

Discussion

The main action mechanism of cisplatin is to interact with the DNA molecule by forming crosslinks or adducts mainly with purine bases. However, its ability to bind to various proteins in the cell generates complex resistance mechanisms that involve genetic and epigenetic changes, as well as variation in protein functionality. This variation includes alterations in the apoptotic signaling pathways,[16] which ultimately manifest themselves in cell structure and function.

Morphological changes associated with cell shape, size, and appearance have been described in various cell lines exposed to cisplatin.[17] Assays on the SiHa cell line showed morphological changes that were maintained throughout the resistance generation process, as well as physiological damage generated as a consequence of exposure to the chemotherapy drug compared to the controls, which preserved their characteristic epithelial morphology with high nuclear/cytoplasmic ratios and conspicuous nucleoli.[18] Morphological changes were observed in the cells that were exposed to cisplatin, with an increase in fragility. Such changes suggest that the cells were entering into oxidative stress processes. In addition, changes in size and cell contraction allow us to suggest that apoptosis is the type of death involved in cisplatin treatment, as reported by *Wang et al.*[19]

Another change that was present throughout the resistance generation process was the appearance of cytoplasmic granules. Once exposed to cisplatin, these granules did not disappear, even when the cells generated the resistant phenotype. It is suggested that these granules may appear due to different mechanisms of resistance to the chemotherapy drug, such as intracellular reduction of reactive cisplatin by inactivation of thiol-containing proteins.[16] Given that cisplatin can bind to other therapeutic targets upon entering cells, if it binds to thiol-containing proteins like methionine or metallothionein, it can deplete intracellular antioxidant reserves, reducing the availability of reactive cisplatin.[20] By binding to these proteins, cisplatin does not fulfill its function of damaging DNA, but neither is it expelled from the cell for detoxification, and this

cisplatin inactivation is evidenced in the form of cytoplasmic granules.[21] Another possible explanation for the appearance of granules in the cytoplasm may be the generation of endoplasmic reticulum stress. This leads to the unfolded protein response (UPR), whereby unfolded proteins accumulate in both the cytoplasm and the endoplasmic reticulum (ER). This can be seen in the granules observed in the cytoplasm of the resistant phenotype.

A great number of proteins are folded in the ER lumen to then be secreted and transported to the organelle, where they will ultimately perform their function. However, since the ER is one of cisplatin's cytoplasmic targets, its function may be affected. Consequently, inadequate folding of proteins may occur, which are retained in the ER for subsequent degradation and thereby reestablish cellular homeostasis or induce apoptosis.[22] When there is an accumulation of unfolded or misfolded proteins in the ER, ER stress is produced, and the UPR mechanism is activated, which indicates that the ER stress could not be mitigated. At this point, UPR activation may reduce the protein load in the ER, temporarily inactivating protein synthesis and activating gene transcription programs that increase protein folding, or, if cellular homeostasis is not achieved, ER-mediated apoptosis is induced. On the other hand, UPR activation may favor the development of cisplatin resistance in cells by activating still unclear mechanisms to protect them from ER-mediated apoptosis.[23] Additionally, *Chen et al*. (2011) suggest that UPR has a cytoprotective function in cisplatin-treated cells, which depends on cell autophagy,[24] since its activation by ER stress has been demonstrated to be a defense mechanism for cell survival.[25] These unfolded, or misfolded proteins accumulate in the ER lumen and could be expelled from the ER and precipitate, or simply fail to degrade and accumulate to form the granules observed in cisplatin-resistant cells.

In healthy adult mammals, the expression of Kv10.1 is restricted mainly to cells of the nervous system, and to date, no reports have been found on the expression of the Kv10.1 protein in healthy cervical cell lines. However, some results observed in the present study (data not shown) confirmed that by obtaining a similar expression of this potassium channel in cervical cancer cells and in mouse brain tissue, it is accepted that SiHa cells fundamentally present an alteration in Kv10.1 and p53 expression. This is because they

both have been described as oncogenic precursors associated with processes such as altered apoptosis, angiogenesis, and cell volume control.[10,26,27] However, the effect of cisplatin on Kv10.1 and p53 expression when generating resistance has not yet been described in cervical cancer SiHa cells, although similar studies with cell lines of other types of gynecologic cancer have not reported an increase in the expression of p53, concluding that the cytotoxicity induced *in vitro* by cisplatin in ovarian cancer cells is independent of p53.[27]

Additionally, a study with ovarian cancer cells transfected with HPV-16 demonstrated that when the E6 protein is expressed by the presence of the virus, it tends to degrade or inhibit p53.[27,28,29]

This result is in line with those obtained in this study, considering the significant decrease in p53 expression when adding 0.05 µg/mL and 0.5 µg/mL of cisplatin during the generation of resistance (fig. 3.B).

Although the relationship between p53 and Kv10.1 expression in cancer cell lines without drug exposure tends to work in opposite directions,[26] in this study, cisplatin exposure was observed to interfere with the regulatory mechanism of these two proteins, resulting in similar protein expressions during the generation of resistance to the chemotherapy drug (fig. 3.A and 3.B).

The significant differences found in the behavior of the proteins at different doses of cisplatin showed a decrease in Kv10.1 and p53 expression during exposure to cisplatin concentrations of 0.05 µg/mL and 0.5 µg/mL, this being the first report in a cervical cancer cell line. Given the previously described cell membrane morphological changes, we infer that such changes are associated with drug exposure. We propose that, upon the exposure of the cells to a first dose of cisplatin, the Kv10.1 channel is affected, decreasing its expression as a rapid response. It is possible that one or more resistance mechanisms are generated, resulting in the resistant phenotype. We suggest that the cell has other mechanisms of rapid response to small doses of cisplatin that generate resistance without significantly altering the expression of its protein machinery. However, at high doses, there is evident alteration and difficulty in recovering.

The mechanisms of cellular regulation related to the expression of the Kv10.1 channel in gynecologic cancers are related to the modulation of cisplatin resistance as reported in tissue samples of patients with ovarian cancer treated with this chemotherapy drug.[29] However, our results do not show an increase in the expression of Kv10.1 associated with increased resistance. Therefore, it is suggested that the expression of the potassium channel is related to the expression of the p53 protein, considering the similar behavior of both proteins. The decrease in p53 expression is in correspondence with that reported by *Yazlovitskaya et al.* (2001), who demonstrated degradation or inhibition of p53 through the expression of the E6 protein in ovarian cancer cells transfected with HPV-16.[28] This degradation may have no impact or have a negative impact on Kv10.1 expression.

Additionally, the variable expression of Kv10.1 could be due to the cell exposure times at each stage. The concentrations used in this study were taken from a study by *Wen et al.*[14] However, the exposure times of the cells to the different concentrations of cisplatin in our study were lower than those used in their work. This was because a minimum of 80% confluence was required to be reached before increasing the exposure concentration. This confluence was reached in a matter of weeks with the first doses and not months, as reported by Wen *et al.*,[14] who exposed the cells to cisplatin for one month at a concentration of 0.1 µg/mL, 2 months at 0.5 µg/mL, 3 months at 1.0 µg/mL, 2 more months at 1.5 µg/mL, and finally stayed at 2.0 µg/mL of cisplatin for the following 30 passages. Additionally, the first concentration of cisplatin used in the study was 0.1 µg/mL. This was not tolerated by the SiHa cells used in our work, since all cells showed cell death in the first 24 h of exposure to the chemotherapy drug (data not shown). Therefore, the dosing in the present study started at 0.05 µg/mL.

Apart from the above arguments, the exact reason for there being no relationship between increased resistance and increased Kv10.1 protein expression, as reported in other studies, is unknown.[7,8,9] However, based on the results reported here and considering that there are no similar reports in cisplatin-resistant SiHa cells, it could be expected that increased time of exposure to cisplatin and increased doses lead to generating resistance through mechanisms other than those observed here.

Conclusions

The results obtained in the present study suggest that the response mechanism to cisplatin exposure in SiHa cervical cancer cells differs from that reported for other types of cancer. There was no evidence of an increase in Kv10.1 expression; in contrast, its expression was similar to that observed for the p53 protein. Contrary to what has previously been reported in the literature, Kv10.1 and p53 did not show antagonistic behavior, so the resistance mechanism generated by exposure to cisplatin is unrelated to the activity of both proteins. Furthermore, these results correspond to the first report on the behavior of Kv10.1 and p53 during cisplatin resistance generation for a SiHa cervical cancer cell line, which highlights the need to deepen issues related to the mechanisms of drug resistance in cancer cells in order to develop targeted therapies that help improve the effectiveness of chemotherapy in patients. In addition, conducting similar studies with more sensitive techniques, such as RT-PCR and using normal cervical cells as a control, would be highly recommended to generate a better understanding of the differential expression of these proteins in this type of cancer.

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Conflict of interest

The authors declare that there is no conflict of interest.

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