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TNFRSF10D expression as a potential biomarker for cisplatin-induced damage and ovarian tumor relapse prediction

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ABSTRACT

Among gynecological malignancies, ovarian cancer (OC) presents the most challenging diagnostic scenario. Despite exhaustive efforts, up to 90 % of patients treated with taxane/platinum-based chemotherapy experience relapse, leading to poor survival rates. Identifying new molecular markers that can characterize disease aggressiveness, chemoresistance, recurrence risk, and metastasis is crucial. This study aimed to assess the susceptibility of three ovarian tumor cell lines (TOV-21G, SKOV-3, and OV-90) to cisplatin and paclitaxel, and to investigate the influence of these treatments on the mRNA expression of *TANK*, *RIPK1*, *NFKB1*, *TNFRSF10D*, and *TRAF2*. Among the cell lines, SKOV-3 ovarian adenocarcinoma cells demonstrated the highest resistance to cisplatin treatment (0.125 mg/mL), followed by TOV-21G (0.076 mg/mL) and OV-90 cells (0.028 mg/mL). Regarding paclitaxel treatment, the SKOV-3 cell line exhibited the highest resistance (1.4 μg/mL), followed by OV-90 (1.3 μg/mL) and TOV-21G cells (0.9 μg/mL). Gene expression analysis after paclitaxel treatment remained unchanged; however, after cisplatin treatment, *TNFRSF10D* was observed to be upregulated nearly 100-fold in SKOV-3 compared to all other cell lines studied. SKOV-3 is described as cisplatin and tumor necrosis factor-resistant. Despite the defective signaling of the TNFRSF10D receptor for apoptosis, it can activate the NFKB transcription factor through non-canonical TRAIL signaling, contributing to a pro-inflammatory immune response. In light of this, damage associated with cisplatin increases *TNFRSF10D* expression and may promote cell survival through non-canonical NFKB pathway activation. This suggests that resistance to TRAIL-induced apoptosis in these cells could serve as a promising chemoresistance biomarker in OC.

1. Introduction

Epithelial ovarian cancer (EOC) is one of the most lethal gynecological cancers and a leading cause of cancer death among women. EOC is generally diagnosed at a late stage when the tumor has disseminated throughout the peritoneal cavity, limiting the potential benefits of surgery. Platinum-containing drugs (cisplatin, carboplatin, and oxaliplatin) or taxane compounds (paclitaxel and docetaxel) are highly active agents widely used for the treatment of various gynecological malignancies. However, the development of chemoresistance remains one of the major obstacles in patients with EOC [1].

EOC is not a single disease but presents a diversity of morphological and molecular features, with different histological subtypes. It is classified into type I and type II tumors according to the dualistic model of epithelial ovarian carcinogenesis proposed by Kurman and Shih in 2016 [2]. Type I carcinomas include endometrioid, clear cell, low-grade serous, and mucinous carcinomas, with seromucinous carcinomas and malignant Brenner tumors being infrequent. Type II carcinomas primarily consist of high-grade serous carcinoma, carcinosarcoma, and undifferentiated carcinoma [2].

Therefore, it is necessary to develop strategies that can efficiently eliminate cancer cells with lower side effects and higher specificity. Among novel therapeutic approaches, the selective induction of programmed cell death (apoptosis) in tumor cells is highly desirable. TNF superfamily member 10 (TNFSF10 or TRAIL) is a member of the TNF superfamily that can favorably induce apoptosis in a variety of primary

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tumor cells. TRAIL has four membrane-bound receptors. TRAILR-1 (also known as DR4 or TNFRSF10A) and TRAILR-2 (also known as DR5 or TNFRSF10B) can induce apoptosis via their death domains (DD). On the other hand, TRAILR-3 (also known as DCR1 or TNFRSF10C) and TRAILR-4 (also known as DCR2 or TNFRSF10D) do not have the functional DD needed to trigger apoptosis [3,4].

According to the literature, TRAILR-4 contains a truncated domain that can induce NFKB activation but not apoptosis upon binding of TRAIL to TRAILR-4. Receptor-interacting serine/threonine protein kinase 1 (RIPK1) is also involved in NFKB activation by leading the IKK complex to its degradation and NFKB nuclear translocation. NFKB then promotes the transcription of genes that protect cells from apoptosis and induce cell survival and proliferation, thereby increasing the malignant properties of the tumor [3–5].

In this paper, we investigated the roles of *TANK* (TRAF family member associated NFKB activator), *RIPK1* (receptor-interacting protein kinase 1), *NFKB1* (nuclear factor kappa B subunit 1), *TNFRSF10D* (TNF receptor superfamily member 10d), and *TRAF2* (TNF receptor-associated factor 2) in the mechanisms of susceptibility and resistance to cisplatin and paclitaxel in cell lines derived from ovarian tumors (OV-90, SK-OV-3, and TOV-21G).

2. Material and methods

2.1. Cell culture

The cell lines OV-90 (serous adenocarcinoma - CRL-11732™), SKOV-3 (adenocarcinoma of the ovary derived from ascites - CRL-HTB-77™), and TOV-21G (clear cell carcinoma - CRL-11730) were purchased from the American Type Culture Collection (ATCC®). The OV-90 cell line was cultured in a 1:1 mixture of MCDB 105/199 Medium supplemented with 15 % bovine fetal serum (BFS) and 1 % 0.2 M L-glutamine. The SKOV-3 cell line was grown in McCoy's medium supplemented with 10 % fetal bovine serum and 1 % 0.2 M L-glutamine, while the TOV-21G cell line was cultured in DMEM High Glucose Medium supplemented with 15 % FBS and 1 % 0.2 M L-glutamine. Cells were incubated at 37 °C in a humidified atmosphere enriched with 5 % CO_2. The experiments were carried out following a specific passage number.

2.2. Cell viability assay

OV-90, SKOV-3, and TOV-21G cell lines were seeded into 96-well plates at a density of 2.5×10^4 cells/well and incubated for 24 h in the presence of cisplatin or paclitaxel. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT) [6,7]. Absorbance was measured on a spectrophotometer (SpectraMax® M5e) at 550 nm. The individual survival of treated cells was calculated by dividing the absorbance value of the treated cells by the mean of the control, then multiplying by 100. The IC_{50} was determined using the statistical program Dr. Fit [8].

2.3. Extraction of total RNA and reverse transcription

Total RNA was extracted from tumor cell lines in culture before and after treatment with cisplatin or paclitaxel using the TRIzol® reagent (Invitrogen™, USA) following the manufacturer's instructions. The quantification of the total RNA extracted from cells was performed using a NanovueTM Plus Spectrophotometer (GE Healthcare Life, USA). The integrity of the total RNA extracted was evaluated by 1 % agarose gel electrophoresis. A total of 2 μg of total RNA was treated with the RNase-Free DNase Set (Qiagen) kit, and complementary DNA synthesis (cDNA) was performed using the M-MLV Reverse Transcriptase® kit (Sigma) according to the manufacturer's instructions.

2.4. Quantitative real time PCR (qPCR)

Transcript analysis of *TANK, RIPK1, NFKB1, TNFRSF10D*, and *TRAF2* (Table 1) was conducted using Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) with TaqMan® Assays (Applied Biosystems™) and TaqMan® Universal Master Mix (Thermo Fisher Scientific™), following the manufacturer's specifications. *TBP* gene was used as endogenous control. Transcript analysis of *TP53* (Table 2) was performed using Power SYBR™ Green PCR Master Mix (Applied Biosystems). The *RPS26* gene was included as endogenous controls. The reactions were subjected to specific amplification cycles (40 cycles of denaturation at 95 °C for 3 s and annealing/elongation at 64 °C for 30 s) and the dissociation curve. This assay was performed in duplicate. Cycle and data collection were performed by the StepOne ™ Real-Time PCR System (Applied Biosystems ™).

2.5. Data analysis

Gene expression was quantified by relative quantification (RQ) using the $2^{-\Delta\Delta Ct}$ method [9]. Statistical analyses were conducted using SPSS 20 software (IBM). Differences in gene expression was analyzed using ANOVA post hoc LSD. Cell viability results was evaluated using the non-parametric Kruskal-Wallis test. A p-value of < 0.05 was considered statistically significant.

2.6. Visual network pathway analysis

The STRING software version 12 (https://string-db.org/) search tool was used for protein–protein interaction analyses to examine the functional interaction networks between all the targets using Biological Process (Gene Ontology) information.

3. Results

a. Sensitivity to cisplatin and paclitaxel between cell lines

The morphology of the TOV-21G, SKOV-3, and OV-90 cell lines cultured in an adherent monolayer is shown in Fig. 1A. The 50 % inhibitory concentration (IC_{50}) of cisplatin and paclitaxel was determined for each cell line. Statistical analysis revealed a clear distribution of the dose needed to inhibit 50 % of cell growth among the cell lines. After treatment with cisplatin, we determined that the IC_{50} for OV-90, SKOV-3, and TOV-21G was 0.028 mg/mL (± 0.003), 0.125 mg/mL (± 0.025), and 0.076 mg/mL (± 0.001), respectively (Fig. 1B). SKOV-3 cells showed higher survival compared to the OV-90 and TOV-21G cell lines, indicating that SKOV-3 is more resistant to cisplatin treatment. Regarding paclitaxel treatment, the established IC_{50} for each cell line was 0.9 μg/mL (± 0.204) for TOV-21G, 1.4 μg/mL (± 0.195) for SKOV-3, and 1.3 μg/mL (± 0.307) for OV-90.

b. Cisplatin and paclitaxel alter the gene transcription in ovarian cancer cells

Our next step was to evaluate genes that play an important role in the apoptotic process. The gene expression of *TANK, RIPK1, NFKB1, TNFRSF10D,* and *TRAF2* was assessed using qRT-PCR with TaqMan® Gene Expression Assay on each ovarian cancer cell line treated with the

Table 1
Details of TaqMan® gene expression assays used for qPCR.

Gene symbol	Gene name	Assay ID (Sigma, USA)
TANK	*TRAF family member associated NFKB activator*	Hs00370305_m1
RIPK1	*Receptor-Interacting Protein Kinase 1*	Hs01041869_m1
NFKB1	*NFKB1 nuclear factor kappa B subunit 1*	Hs00765730_m1
TNFRSF10D	*TNF receptor superfamily member 10d*	Hs00388742_m1
TRAF2	*TNF receptor associated factor 2*	Hs00184192_m1
TBP	*TATA-box binding protein*	Hs00427620_m1

Table 2
Details of primers used for qPCR with Sybr Green.

Gene symbol	Gene name	Primers sequence (5'-3')	Primers concentration (nM)
TP53	*tumor protein p53*	FW 5'CAGCACATGACGGAGGTTGT3' RV 5'TCATCCAAATACTCCACACGC3'	250FW/150RV
RPS26	*ribosomal protein S26*	FW 5'CGTGCTTCCCAAGCTGTACGTGA3' RV 5'CGATTCCGGACTACCTTGCTGTG3'	200FW/250RV

IC_{50} for cisplatin and paclitaxel, with untreated cells serving as controls (Fig. 2 A–C). The gene expression profile remained unchanged for all three cell lines after paclitaxel treatment. It was observed that the *TNFRSF10D* (*TRAILR-4*) gene is overexpressed in all cell lines treated with cisplatin, with a more pronounced up-regulation in the SKOV-3 cell line. However, changes in the profiles of the *RIPK1, NFKB1,* and *TRAF2* transcripts did not accompany the overexpression of *TNFRSF10D*. Additionally, we observed that the expression of *TANK* in TOV-21G and OV-90 cell lines treated with cisplatin is higher compared to *TRAF2*.

It was observed that the *TP53* gene was up-regulated in the TOV-21G and SKOV-3 cell lines (Fig. 3A) when treated with cisplatin, however, the same target was not amplified in OV-90 cells treated with cisplatin. On the other hand, paclitaxel treatment down-regulated *TP53*

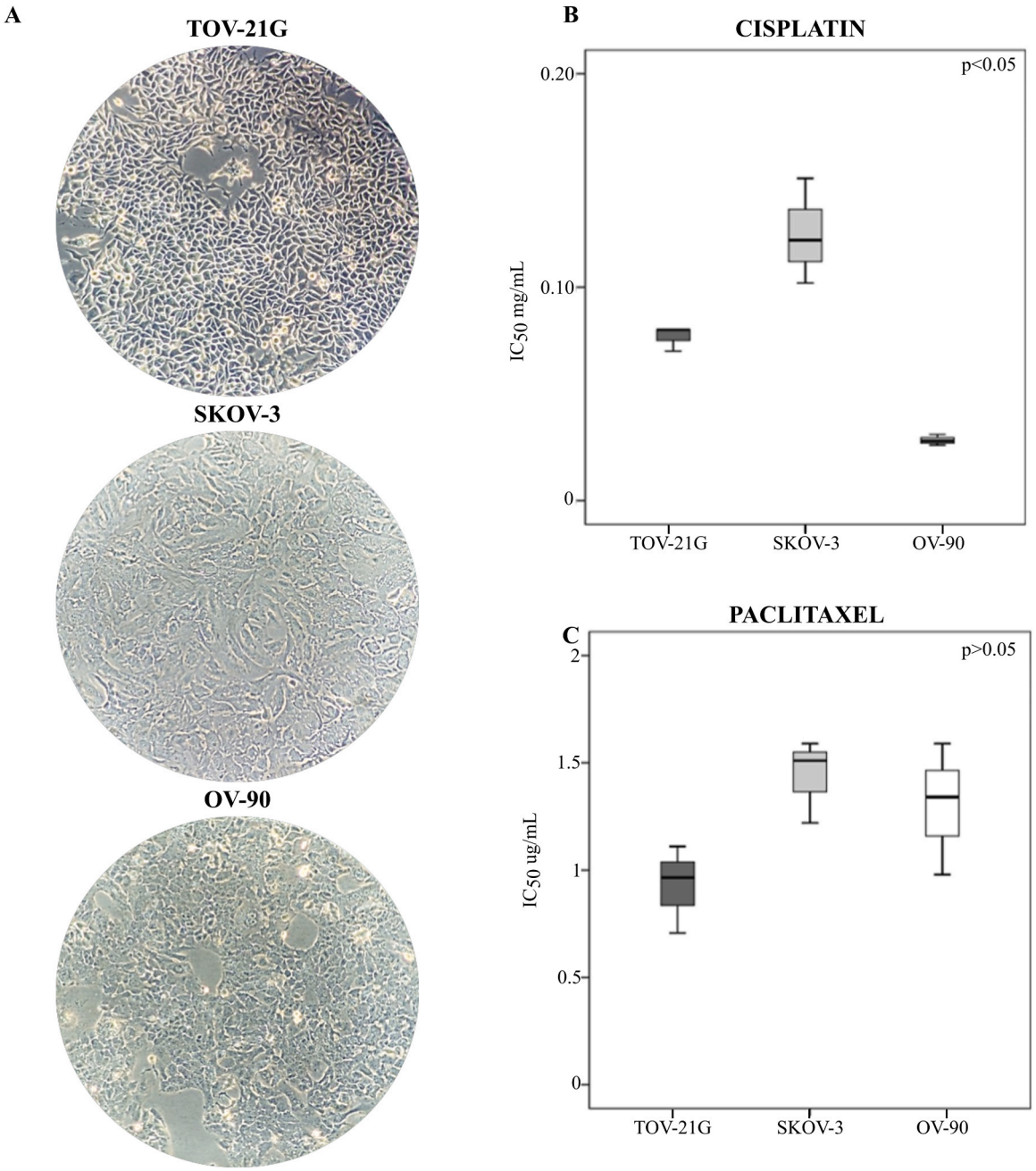

Fig. 1. Cell morphology and IC_{50} values distribution among cell lines. (A) Ovarian cancer cell lines was cultured in adherent monolayer. (B) The SKOV-3 cell line was the most resistant to treatment with cisplatin (0.125 mg/mL ± 0.025), followed by TOV-21G (0.076 mg/mL ± 0.001), and OV-90 (0.028 mg/mL ± 0.003). Significance level = $p < 0.05$. (C) Regarding Paclitaxel treatment, the established IC_{50} for each cell line was TOV-21G (0.9 μg/mL ± 0.204), SKOV-3 (1.4 μg/mL ± 0.195), and OV-90 (1.3 μg/mL ± 0.307). Data were expressed as the mean ± standard deviation (SD). $n = 3$. Significance level = $p < 0.05$.

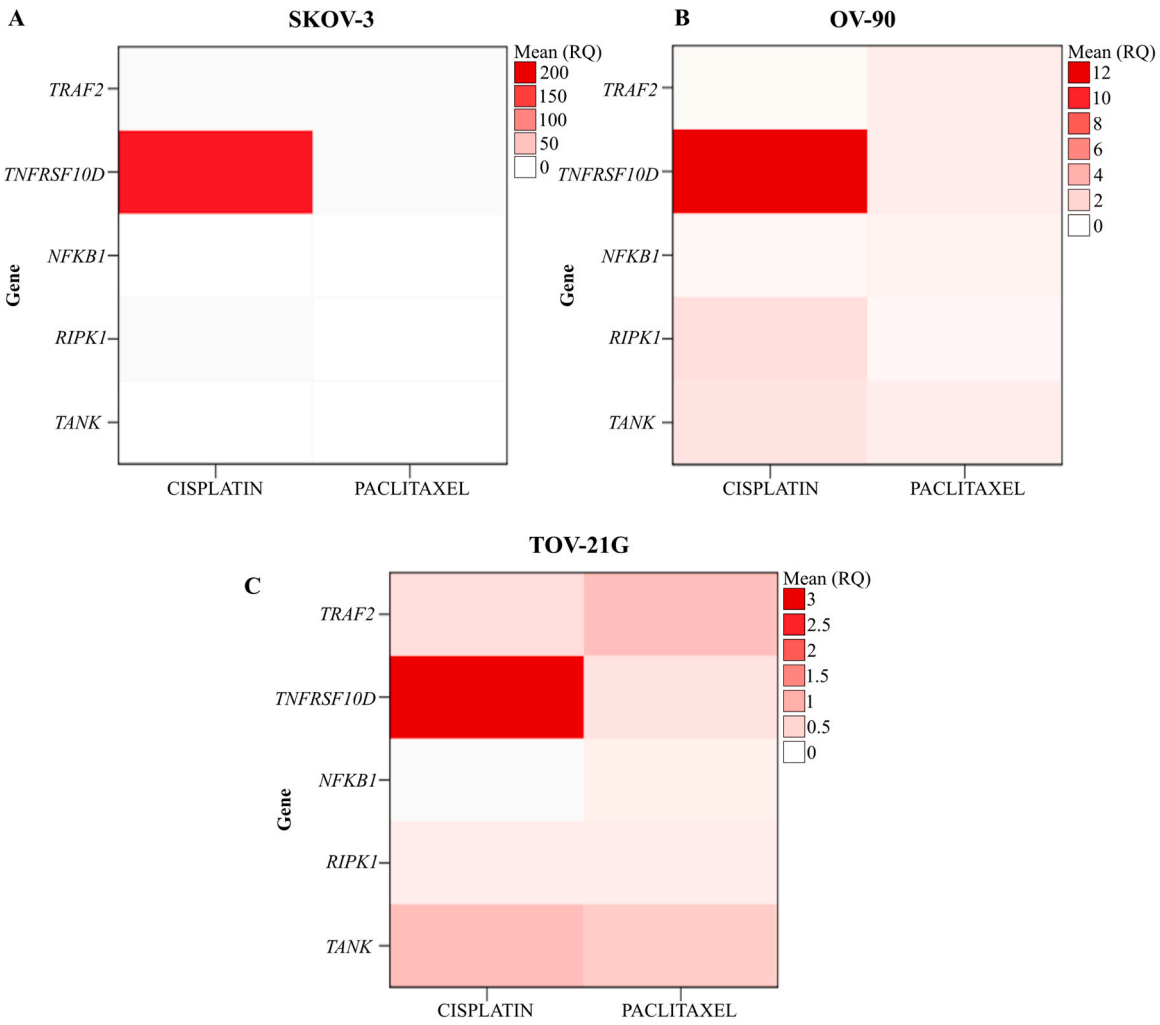

Fig. 2. Cisplatin and paclitaxel alter the gene transcription of *TANK, RIPK1, NFKB1, TNFRSF10D,* and *TRAF2* in ovarian cancer cell lines. (A–C) A notable upregulation of the *TNFRSF10D* gene was observed in all cell lines, with a particularly pronounced increase in the SKOV-3 cell line. To determine relative expression ($2^{-\Delta\Delta Ct}$ method) of the in the samples treated with cisplatin or paclitaxel, we used nontreated samples as calibrators. Data were expressed as the mean ± standard deviation (SD). $n = 2$. Significance level = $p < 0.05$.

expression in SKOV-3 cells, while promoting an overexpression of this gene in TOV-21G and OV-90 (Fig. 3B).

In STRING analyses (Fig. 3C), nodes were clustered by the five biological process in Gene Ontology (GO) that are more attractive to understand the protein-protein interaction (PPI) between TANK, RIPK1, NFKB1, TNFRSF10D, TP53, and TRAF2. These are: (1) Positive regulation of extrinsic apoptotic signaling pathway (red nodes); (2) Negative regulation of I-kappaB kinase/NF-kappaB signaling (blue nodes); (3) Regulation of I-kappaB kinase/NF-kappaB signaling (green nodes); (4) Negative regulation of apoptotic process (purple nodes), and (5) Positive regulation of apoptotic signaling pathway (yellow nodes). Different types of protein protein interactions were observed such as: (1) protein homology; (2) co-expression; (3) gene co-occurrence, and (4) gene neighborhood. We observed that are a strong and confident correlation among the proteins (PPI enrichment p-value: 0.000249).

4. Discussion

According to the molecular classification proposed by Kurman and Shih (2016), epithelial ovarian cancer (EOC) can be categorized into two groups, namely, type I and type II. Type I tumors are characterized by mutations in *KRAS*, *BRAF*, *ERBB2*, *PTEN*, *CTNNB1*, and *PIK3CA*, with *TP53* mutations being uncommon. On the other hand, type II tumors exhibit a high frequency of *TP53* mutations [2].

According to ATCC, TOV-21G cells represent a clear cell carcinoma (grade 3, stage III) with a genetic profile featuring mutations in *KRAS* and *PIK3CA*, thus falling under the classification of Type I tumors as per the dualistic classification proposed by Kurman and Shih [2]. The SKOV-3 cell line is classified as an adenocarcinoma derived from metastatic ascites and exhibits mutations characteristic of both Type I and Type II tumors. Conversely, the OV-90 cell line is classified as an ovarian malignant papillary serous adenocarcinoma (grade 3, stage IIIC) and can be categorized as type II.

The IC_{50} concentration achieved for cisplatin in SKOV-3 cells was 2–4 times higher than in TOV-21G and OV-90 cells, supporting the characterization of platinum resistance for this cell line [10–12]. The diversity observed in the cytotoxicity effect of cisplatin among the three cell lines is likely due to their differential genetic profiles. IC_{50} values for the three cell lines after paclitaxel treatment remained similar without significant differences. The efficacy of paclitaxel is not associated with platinum resistance in ovarian cancer cell lines [13].

Tumor heterogeneity is characterized by the existence of various cell subpopulations or clones that possess distinct genotypic and phenotypic profiles. This diversity plays a pivotal role in the growth, progression, and development of therapeutic resistance in tumors [14–19]. Considering the distinct genetic profiles described for EOC, these three cell lines serve as satisfactory models for studying chemotherapy response. Chemoresistance leads to therapeutic failure and death in over 90 % of

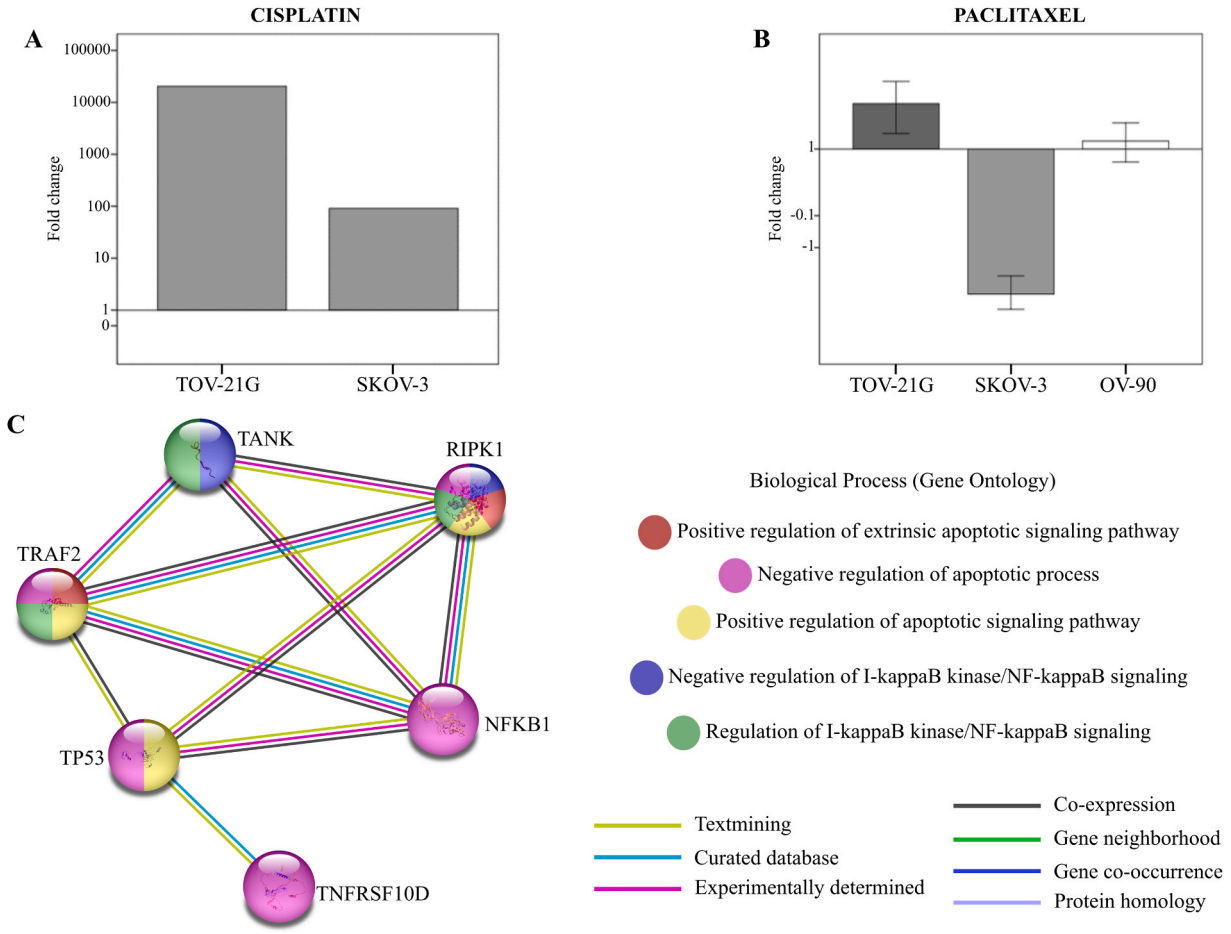

Fig. 3. Cisplatin and Paclitaxel induce transcriptional changes in the *TP53* gene profile in ovarian cancer cells. (A) Cisplatin induces up-regulation of *TP53* mRNA in TOV-21G and SKOV-3 cells. (B) Paclitaxel treatment results in down-regulation of the *TP53* gene in SKOV-3 cells, while increasing its transcription in TOV-21G and OV-90 cell lines. (C) Nodes in STRING (v.12) analyses were clustered based on five biological processes in Gene Ontology (GO), and edges represent different types of protein-protein interactions. A strong and confident correlation was observed among the proteins (PPI enrichment p-value: 0.000249). Data were expressed as the mean ± standard deviation (SD). $n = 2$. Significance level = $p < 0.05$.

patients with advanced-stage disease. A poor prognosis is often associated with relapse and metastasis. Therefore, evaluating gene expression profiles is widely used to identify predictive markers that can aid in optimal treatment selection [20]. Additionally, the discovery of biomarkers associated with chemoresistance holds promise for clinical practice, as none of the currently available drugs seem to significantly improve survival in patients who are refractory to platinum-based compounds [21].

In cisplatin treatment, overexpression of *TNFRSF10D* was observed in all three cell lines, most significantly in the SKOV-3 cell line. TRAIL-R4 is a member of the TNF receptor superfamily and possesses a truncated cytoplasmic death domain, rendering it unable to induce TRAIL-induced cell apoptosis. However, TRAIL-R4 may contain interactive motifs for TRAF in its defective intracellular domain, enabling it to mediate a signaling complex and activate other pathways [22]. TRAILR-4 can recruit proteins such as TRAF2 and RIPK1 to its intracytoplasmic domain and activate pathways related to tumor progression, such as the NFKB pathway [23].

In this study, we observed that *TNFRSF10D* overexpression did not coincide with an increase in *RIPK1, TRAF2,* and *NFKB1* mRNA expression. In a previous investigation, Suliman et al. [24] demonstrated that the increase of TRAILR-4 expression alone is insufficient to activate the NFKB pathway, as it depends on the presence of its ligand (TRAIL). TANK plays a dualistic role in the regulation of the NFKB pathway, as it can participate in activation through interaction with the IKK complex, or competitively interact with TRAF2, resulting in NFKB inhibition [25].

Duiker et al. [26] documented that pre-exposure of ovarian tumor cell lines to cisplatin increases the expression of TRAILR-1 and TRAILR-2, thereby rendering cells more sensitive to apoptosis induced by recombinant human TRAIL. Additionally, in cervical tumor cell lines where TRAILR-4 was downregulated, the susceptibility to apoptosis following exposure to cisplatin and recombinant human TRAIL was heightened, suggesting that apoptosis mediated by recombinant human TRAIL depends on the equilibrium between death receptors and decoy receptors [27].

Previous studies in our group demonstrated a correlation between the overexpression of mRNA from the decoy receptor TRAILR-3 and tumor samples of metastatic ovarian cancer, compared to primary tumor samples [28]. These data underscore the association between decoy receptors and advanced metastatic stages and relapse. Cells can activate numerous survival mechanisms, and the overexpression of *TNFRSF10D* may be related, especially considering that SKOV-3 is a metastatic cell line derived from ascites and is described as resistant to cisplatin and TNF [12].

The tumor suppressor TP53 (p53) plays a crucial role in tumor suppression by regulating the transcription of various genes associated with cell death, particularly apoptotic cell death. Among its pro-apoptotic transcriptional targets are *TNFRSF10A*, *TNFRSF10B*, *TNFRSF10C*, and *TNFRSF10D* [29–31]. Our results indicate that SKOV3, with a *TP53* mutation, exhibits a higher level of *TNFRSF10D* expression than TOV-21G, which has a *TP53* wild type [32].

Our current discovery that *TNFRSF10D* gene expression is dependent

on cisplatin and is a p53 target gene in ovarian cancer cell lines provides compelling evidence that this receptor holds promise as a relapse biomarker. This is particularly significant as it can hinder TRAIL-induced apoptosis by inducing NFKB activation. Furthermore, drugs designed to activate death receptors (or block decoy receptors) may be employed to eliminate tumor cells resistant to conventional cancer therapies.

5. Conclusion

In spite of advancements in diagnostic and therapeutic methodologies, the overall survival rate for the majority of ovarian cancer patients remains low. Consequently, there is considerable interest in identifying novel biological targets that can enhance early diagnosis and/or contribute to improved prognosis and predictive therapeutic responses. In this context, the overexpression of *TNFRSF10D* in ovarian cancer cell lines treated with cisplatin may serve as a potential biomarker in ovarian cancer.

CRediT authorship contribution statement

Luciana Maria Silva: Writing – original draft, Supervision, Formal analysis, Conceptualization. **Agnaldo Lopes da Silva Filho:** Writing – original draft, Conceptualization. **Letícia da Conceição Braga:** Conceptualization. **Nikole Gontijo Gonçales:** Formal analysis, Data curation. **Bryan Ôrtero Perez Gonçalves:** Writing – review & editing, Writing – original draft.

Patient consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Funding

No funding was received.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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