

The Impact of Progesterone Receptor Expression on Platinum Sensitivity and Survival Outcomes in Patients With Ovarian Clear Cell Carcinoma

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Research Article

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Abstract

Background

Ovarian clear cell carcinoma (OCCC) is considered to be a relatively platinum-resistant malignancy. In the present study, we investigated the impact of progesterone receptor (PR) expression on platinum sensitivity and survival outcomes in patients with OCCC.

Methods

We retrospectively reviewed 80 patients with OCCC who underwent surgery followed by adjuvant chemotherapy, and analyzed PR expression by immunohistochemical staining. The PR expression was quantified using the H-score. The platinum sensitivity and survival outcomes were compared between those with a weak and strong PR expression. Cisplatin viability experiments were performed in OCCC cell lines (TOV-21G) with different PR expressions.

Results

Among the 80 patients, 66 and 14 were considered to have platinum-sensitive and platinum-resistant disease, respectively. The mean PR H-score of the platinum-sensitive tumors was significantly higher than that of the platinum-resistant tumors ($p=0.002$). Although there were no significant differences in progression-free and overall survival between the patients with high and low PR expressions, the patients with a high PR expression had a trend towards better survival. In cell models, PR protein was weakly detectable in TOV-21G cells. Through transfection of PR gene, TOV-21G cells were shown to have a strong PR expression by Western blot analysis. After treating the TOV-21G cells with cisplatin, we found that the overexpression of PR enhanced cisplatin cytotoxicity.

Conclusions

The patients in this study with a strong PR expression were associated with better platinum sensitivity and survival, and this was compatible with our experimental findings. The value of PR as a tumor sensitizer to cisplatin in OCCC should be further investigated.

Background

In the United States, approximately 22,530 patients were newly diagnosed with ovarian cancer and approximately 13,980 patients died from this disease in 2016, representing the fifth leading cause of cancer-related deaths in women [1]. According to the Taiwan Cancer Registry, there were 1,507 newly diagnosed ovarian cancer patients and 656 deaths in Taiwan in 2016, representing the seventh leading cause of cancer-related deaths in women.

Most ovarian malignancies are epithelial ovarian cancer (EOC), however differences in the incidence of EOC subtypes have been reported in different areas. Ovarian clear cell carcinoma (OCCC) is unique at

cellular and molecular levels. Endometriosis is a precancerous lesion that has been associated with a 3-fold increased risk of OCCC, and the incidence of OCCC ranges from 13–28% in Asia, which is much higher than in Western countries (5–10%) [2–5]. According to a report from the Taiwan Cancer Registry, OCCC accounted for 14–18% of all cases of EOC from 2012–2016. OCCC is more commonly detected at an early stage and has a good prognosis. However, it is considered to be platinum resistant in the advanced stage, and the prognosis is worse than high-grade serous carcinoma [6–9]. Therefore, novel treatments are urgently needed, and many molecular targeted therapies and immunotherapies are currently being studied.

The presence of sex steroid hormone receptors in EOC tissue suggests that hormones may play a role in the origin and promotion of this disease [10]. Epidemiological evidence strongly suggests that progesterone-containing contraceptives and pregnancy appear to provide a protective effect against EOC, indicating that progesterone receptor (PR) may play a role in predicting the prognosis [11]. Although previous studies have reported an association between a strong PR expression and improved disease-specific survival in patients with ovarian high-grade serous and endometrioid cancer, the results in OCCC have been conflicting due to limited sample sizes and statistical power [12, 13]. However, differential expressions of PR from atypical endometriosis to OCCC have been reported, suggesting its role in predicting prognosis [14, 15]. As the association between PR status and chemosensitivity has not been investigated before, the aim of this study was to evaluate the associations among PR expression, platinum sensitivity, and survival in OCCC patients and cell line models.

Materials And Methods

Patients

We retrospectively reviewed patients with OCCC between January 2008 and December 2016 in Kaohsiung Chang Gung Memorial Hospital who underwent primary surgery followed by more than 4 courses of adjuvant chemotherapy. The stages were assigned according to the International Federation of Gynecology and Obstetrics (FIGO) 2014 system. The study cases were selected based on the availability of tissue for immunohistochemical (IHC) staining. Patients were excluded if sufficient tissue samples were not available, they had not undergone chemotherapy or received fewer than 4 courses of chemotherapy, did not have regular follow-up data, or had double cancers. Clinical data including demographics, FIGO stage, carbohydrate antigen-125 (CA-125) level, concurrent endometriosis (defined as endometriosis presenting at the same site of cancer), platinum sensitivity, PR expression, and survival time were collected from clinical records. Platinum sensitivity was classified as being either resistant or sensitive according to the time of relapse (6 months as the cut-off) since finishing first-line chemotherapy. Clinical parameters including PR expression were compared between the platinum-sensitive and platinum-resistant groups. The study protocols were approved by the Institutional Review Board of Chang Gung Memorial Hospital (approval number: 201601487B0C503).

Immunohistochemistry analysis

Paraffin-embedded ovarian tissues were sliced into 3- μ m-thick sections, deparaffinized in xylene, and rehydrated through an alcohol gradient. Activity of endogenous peroxide was blocked with 3% H₂O₂ for 15 min. The slides were then treated with 1x citrate buffer pH 6.0 (SIGMA, C9999) for 17 min in a microwave oven for antigen retrieval, and then incubated with the primary PR antibody (1:200, ThermoFisher, MA1-411) at 4°C overnight, which can detect both the A and B form of PR. IHC staining was performed using an UltraVision Quanto Detection System HRP kit (ThermoFisher, TL-060-QHL) and DAB Quanto reagent (ThermoFisher, TA-125-QHDX) according to the manufacturer's instructions. Finally, the sections were counterstained with hematoxylin and mounted with aqueous mounting media. The slides were visualized at 200X magnification, and PR staining was scored using the H-score method (range 0-300) obtained by multiplying the tumor nuclei cell intensity (on a scale of 0 to 3) by the percentage of positive tumor nuclei cells (on a scale of 0 to 100) [16]. One pathologist (Lan J) blindly scored all of the cases.

PR-overexpressing ovarian cancer cell lines

The plasmids pcDNA3-hPR-A (Cat.No.#89119) and pcDNA3-PRB (Cat.No.#89130) were purchased from Addgene. TOV21G cells were seeded in a 6-well plate at 3×10^5 cells per well for 24 h, and transfection was carried out using Lipofectamine 2000 reagent (Invitrogen, Cat.No.#11668019) according to the manufacturer's instructions. After transfection for 48 h, the cells were harvested for Western blot and cell viability analyses.

Cell viability assay

PR-overexpressing cells were seeded in a 96-well plate at 5000 cells per well, and incubated at 37°C overnight before drug treatment. The cells were treated with different concentrations of cisplatin for 24 h, 48 h, 72 h, and 96 h. PrestoBlue Cell Viability Reagent (10:1) (Invitrogen, A13261) was then added and allowed to incubate at 37°C for 2–3 h. The absorbance at a wavelength of 570–600 nm was measured using a SpectraMax ABS Absorbance Microplate Reader (Molecular Devices). The viability of cells was expressed as a percentage of absorbance in treated wells relative to that in untreated (control) wells.

Western blot analysis

Cell proteins were lysed in PRO-PREP Protein Extraction Solution (iNtRON, Cat.No.#17081.1), and the proteins of the lysates were quantified with a BCA Protein Assay kit (Merck Millipore, 71285-3). After the proteins had been separated by 8% – 10% SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, IPVH00010), the membranes were blocked with 5% non-fat milk in Tris Buffered Saline (SIGMA, T5912) containing 0.1% Tween-20 for 30 min at room temperature, and probed with specific primary antibodies against PR (1:2000, ThermoFisher, MA1-411) and β -actin (1:10000, Merck Millipore, A5441) at 4°C overnight. The secondary antibody was conjugated with horseradish peroxidase (HRP) (1:8000, Merck Millipore, A9044) for 90 min at room temperature. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, WBKLS0500) and exposed to X-ray film for autoradiography.

Statistical analysis

Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of PR H-score to predict platinum sensitivity and survival. Progression-free survival (PFS) and overall survival (OS) were defined as the interval from the date of diagnosis to the first evidence of progression and death, respectively. Comparisons of median and mean values were performed using the two-sample *t*-test. Frequency distributions between categorical variables were compared using the chi-square or Fisher's exact test. Multivariate Cox proportional hazards analysis was used to identify the most significant independent prognostic factors for PFS and OS. Actuarial rates of survival were estimated with the Kaplan-Meier method, and statistical differences between groups were examined using the log rank test. The Student's *t*-test was used to assess statistical significance between the control and PR-A/PR-B treatment groups and the PrestoBlue assay was used to assess differences between control and PR-A/PR-B groups in the difference concentration of cisplatin treatment using the cell line TOV-21G. Data management and analysis was performed using SPSS software for Windows version 22 (IBM, Armonk, NY, USA). A *p* value less than 0.05 was considered to indicate statistical significance.

Results

Clinical data

A total of 592 ovarian cancer patients were identified during the study period. We excluded 481 patients who had a non-OCCC histology, 15 patients who did not undergo chemotherapy or had received fewer than 4 courses of chemotherapy, 9 patients due to insufficient tissue samples for IHC staining, and 7 patients with inadequate follow-up data or double cancers. Finally 80 patients met the eligibility criteria and constituted the study cohort. The mean age at diagnosis was 48.9 years (range, 28–61 years), and the median follow-up time was 50 months (range, 5–132 months). The basic characteristics of the patients are listed in Table 1. Among the 80 patients, 66 and 14 cases were defined as having platinum-sensitive and platinum-resistant disease, respectively. The platinum-sensitive patients were significantly associated with a higher rate of concurrent endometriosis and higher mean PR H-score (Table 2). Positive PR staining (H-score > 1) was noted in 30 patients (37.5%). Based on the ROC curve analysis, the optimal cut-off value of PR H-score to predict platinum sensitivity and survival was 50 (area under the curve 0.64, 95% [confidence interval] CI: 0.51–0.77). An H-score of 50 or higher was defined as a strong PR expression (13 patients), and an H-score lower than 50 as a weak PR expression (67 patients). There were no significant differences in the distribution of clinicopathological variables between the weak and strong PR expression groups (Table 3). Typical examples of IHC staining of PR are shown in Fig. 1. Univariate analysis demonstrated significantly better 5-year PFS and OS for the patients with early FIGO stage (I/II), concurrent endometriosis, and pretreatment CA125 level lower than 35 U/mL, while only trends were noted in the strong PR expression group. Multivariate Cox regression analysis showed that only advanced FIGO stage was significantly associated with worse PFS (hazard ratio [HR] 2.427; 95% CI 1.004–5.869) and OS (HR 3.22; 95% CI 1.27–8.161) (Table 4). Because FIGO stage was the only independent factor predicting survival, we further investigated the correlation between PR expression and FIGO stage. We

found a stepwise decrease in PR expression from FIGO stage I to IV, although without significance (Table 3).

Table 1
Clinicopathological characteristics of all patients (N = 80)

Age, mean (SD)	48.9 (10)
Follow-up, months, median (range)	50 (5-132)
FIGO stage, n (%)	44 (55)
I	12 (15)
II	20 (25)
III	4 (5)
IV	
Menopause, n (%)	41 (51.2)
Yes	39 (48.8)
No	
Parity, n (%)	31 (38.8)
Nulliparous	49 (61.2)
≥1	
Pretreatment CA125, n (%)	11 (13.8)
<35 U/mL	59 (73.8)
≥35 U/mL	
Concurrent endometriosis, n (%)	19 (23.8)
Yes	61 (76.2)
No	
PR expression, n (%)	30 (37.5)
Yes	50 (62.5)
No	
PR H-score, mean (SD)	18.95 (34.73)
Platinum sensitivity, n (%)	66 (82.5)
Sensitive	14 (17.5)
Resistant	
<i>CA</i> Carbohydrate antigen; <i>FIGO</i> International Federation of Gynecology and Obstetrics; <i>PR</i> Progesterone receptor	

Table 2
Factors associated with platinum sensitivity in the patients with ovarian clear cell carcinoma

Factors	Platinum-sensitive n = 66	Platinum-resistant n = 14	P value
Age (mean), years	48.3	51.7	0.441
Menopause, n (%)	32 (48.5)	9 (64.3)	0.283
Parity ≥ 1 , n (%)	41 (62.1)	8 (57.1)	0.728
Concurrent endometriosis, n (%)	19 (28.8)	0 (0)	0.033
Pretreatment CA125 (mean), U/mL	537	709	0.825
PR positive, n (%)	29 (43.9)	4 (28.6)	0.376
PR H-score (mean)	21.9	5	0.002
PR H-score ≥ 50 , n (%)	13 (19.7)	0 (0)	0.110
<i>PR</i> Progesterone receptor			

Table 3
Factors associated with PR expression in the patients with OCCC

Factors	PR ^{weak} (n = 67)	PR ^{strong} (n = 13)	p value
Age (mean)	49.49	45.85	0.975
Menopause, n (%)	35 (52.2)	6 (46.2)	0.688
Parity ≥ 1 , n (%)	42 (62.7)	7 (53.8)	0.549
FIGO stage			0.588
I (n = 44) (% within stage)	35 (79.5)	9 (20.5)	
II (n = 12) (% within stage)	10 (83.3)	2 (16.7)	
III (n = 20) (% within stage)	18 (90.0)	2 (10.0)	
IV (n = 4) (% within stage)	4 (100)	0 (0.0)	
Concurrent endometriosis, n (%)	17 (25.4)	2 (15.4)	0.439
Pretreatment CA125 (mean)	573.7	532.2	0.324
CA Carbohydrate antigen; FIGO International Federation of Gynecology and Obstetrics; PR Progesterone receptor; PR ^{weak} PR H-score < 50; PR ^{strong} PR H-score ≥ 50 ;			

Table 4
Multivariate Cox regression analyses of progression-free and overall survival

Factors	PFS			OS		
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
Menopause	1.324	0.515–3.403	0.560	1.940	0.707–5.318	0.198
Parity (≥ 1 vs. 0)	0.856	0.336–2.180	0.745	1.061	0.386–2.920	0.908
Concurrent endometriosis (yes vs. no)	0.141	0.018–1.084	0.060	0.187	0.024–1.464	0.110
CA125 (≥ 35 vs. <35)	3.252	0.409–25.89	0.265	4.767	0.588–38.65	0.144
PR H-score (≥ 50 vs. <50)	0.369	0.085–1.594	0.182	0.311	0.070–1.391	0.126
FIGO stage (III/IV vs. I/II)	2.427	1.004–5.869	0.049	3.220	1.27–8.161	0.014
<i>CA</i> Carbohydrate antigen; <i>CI</i> Confidence interval; <i>FIGO</i> International Federation of Gynecology and Obstetrics; <i>HR</i> Hazard ratio; <i>OS</i> Overall survival; <i>PFS</i> Progression-free survival; <i>PR</i> Progesterone receptor						

Cell line data

The OCCC cell line TOV-21G was obtained from the American Type Culture Collection (Manassas, VA). According to a previous report, the TOV-21G cell line is highly recommended as a model for OCCC research because of similar molecular and immunologic profiles to typical primary tumors [16]. Staining of PR-A and PR-B was very weak in the TOV-21G cells as shown in Fig. 2A. The TOV-21G cells were transfected with plasmids pcDNA3-PRA and pcDNA3-PRB (TOV-21G-PR-A and TOV-21G-PR-B). After transfection, we selected a stable clone with G418 200 ug/mL. Protein was extracted and subjected to Western blot, which showed an approximate 300% increase in PR-A protein expression in TOV-21G-PR-A compared to TOV-21G cells (Fig. 2A). TOV-21G-PR-B cells also demonstrated a significantly higher level of PR-B protein (210%) than TOV-21G cells (Fig. 2B). To determine the cytotoxic effects of cisplatin on TOV-21G cells with different PR-A and PR-B expressions, we used a PrestoBlue Cell Viability Assay. We found that compared with the control (TOV-21G) cells, the inhibition of viability by cisplatin was significantly greater in both TOV-21G-PR-A and TOV-21G-PR-B cells ($p < 0.01$) at 48 and 72 h (Fig. 2A, 2B).

Discussion

Our results demonstrated that platinum-sensitive tumors were associated with a stronger PR expression both in clinical specimens and cell line models. However, PR status was not significantly associated with PFS or OS in the patients with OCCC.

Previous studies analyzing the expression rates of sex hormone receptors in patients with OCCC have reported a wide range of PR expressions from 0% [17] to 60% [18], with most demonstrating < 10% positivity [19–22]. The percentage of PR-positive patients in the present study was relatively high at 37.5%. The differences among various studies may be due to differences in the PR antibodies used in IHC staining. The most common commercially available PR antibodies include clone 16, clone 636, clone 1A6, clone alpha PR6, and clone 1E2. Although all of these antibodies are thought to be able to recognize both PR-A and PR-B isoforms, a previous study demonstrated that some antibodies failed to detect PR-B in tissue sections when using IHC techniques despite their ability to do so with immunoblot analysis [23]. A possible reason is that some epitopes on PR-B may be masked during heat treatment of formalin-fixed tissue, which results in a certain degree of protein tertiary structural change. Therefore, it is possible that PR expression will not be detected or at least will be underestimated in tissues where PR-B is predominant.

To date, no previous study has evaluated PR expression only in OCCC patients. The largest collaborative study from Sieh et al. reported that PR expression was associated with better high-grade serous and endometrioid carcinoma survival but not mucinous or OCCC [12]. Another meta-analysis of 5685 EOC patients from 28 studies found that PR expression was related to PFS and OS, but that when focusing on studies of serous carcinoma the prognostic role of PR disappeared, which is in contrast to Sieh et al.'s study [13]. This discrepancy may be related to differences in the methodologies of measuring PR and interpreting IHC. For example, we quantified PR status using H-score, which is quite different to Sieh et al. Most previous studies have used a three-tier system: negative, weak, and strong, defined as positive staining in < 1%, 1–50%, and \geq 50% of tumor cell nuclei, respectively. In the present study, we found a stepwise reduction in PR expression from FIGO stage I to IV. Previous studies have reported different expressions of PR, ranging from 100% in atypical ovarian endometriosis to 35% in OCCC, suggesting that a loss of PR may be involved in the etiology and progression of OCCC [14, 15]. As the critical point of PR level change in relation to chemosensitivity or survival is unknown, we quantified PR status and identified an optimal cut-off value. However, even when using this cut-off value, PR was still not a significant prognosticator. There are some possible explanations for this. First, we enrolled many FIGO stage I/II patients. Early-stage OCCC has an excellent prognosis, and even with recurrence the time to relapse may be more than 6 months after completing chemotherapy. Whether these tumors were actually platinum-sensitive remains unclear. Second, although we quantified PR status, the H-score values were not normally distributed. We attempted to stratify the patients by early stage versus advanced stage or with concurrent endometriosis versus without, and we even transformed the values to a certain exponent, however we still failed to overcome this problem. Third, the limited number of study patients after excluding others due to various reasons may also have biased the outcomes.

PR has two isoforms, A and B, and the role of each receptor isoform remains unclear with regards to hormone treatment responsiveness and prognosis. In breast cancer, PR-A-predominant breast tumors are more sensitive to anti-progestin treatment, while PR-B-predominant tumors are associated with advanced disease and poor prognosis [24]. In endometrial cancer, both PR-A and PR-B are associated with less aggressive tumors, however only PR-B predicts a better prognosis [25]. An interesting finding in our study was that the patients with either the A or B PR isoform appeared to be more sensitive to cisplatin, and that the patients with a high total PR expression had a trend towards better survival. The association between hormone receptor status and platinum sensitivity has seldom been investigated in EOC. In one preclinical study using OVCAR-3 cells, Preluso et al. reported that a high PR expression in ovarian cancer cells was associated with decreased PR membrane component-1 (PRMC-1) expression, which in turn increased the effectiveness of cisplatin [26]. The same group later transplanted SKOV-3 cells into nude mice, resulting in the development of an OCCC animal model. They demonstrated that PGRMC1 regulated not only the growth and platinum sensitivity of cultured SKOV-3 cells, but also ovarian tumors derived from these cells [27]. In an endometrial tumor model, they also found that PGRMC1-depleted tumors were more responsive to chemotherapeutic stress compared with PGRMC1-intact control tumors [28]. Taken together, these data support the close relationship between PGRMC-1 expression and chemosensitivity. Given the reported inverse relationship between PGRMC-1 and PR expression [26], our results are consistent with Preluso et al., although further studies investigating the relationship between PGRMC-1 and PR with different expressions of the isoforms are needed.

OCCC is thought to be a platinum-resistant malignant disease. Recent studies have identified specific immune-related molecular profiles such as PD-L1 expression and mismatch repair protein defects in OCCC [29, 30], which in turn has led to the development of a novel therapeutic approach focusing on immunotherapy. Although recent phase I/II results of using pembrolizumab were promising [31, 32], the sample size was small and further phase II/III verification is needed. Until then, clinicians still need to identify which patients may really benefit from traditional chemotherapy.

Conclusions

In conclusion, we demonstrated that tumors with a weak PR expression were associated with greater platinum resistance and probably worse survival both in OCCC patients and cell lines. Ascertaining PR status may allow clinicians to more precisely treat OCCC patients with chemotherapy. Further animal experiments and clinical studies are needed to explore the mechanistic roles of PR in predicting chemosensitivity and disease prognosis in OCCC.

Declarations

Ethics approval and consent to participate

Institutional Review Board of Chang Gung Memorial Hospital provided ethical approval for the current study (201601487B0C503). Informed consent for use of retrospective patient case data was waived. All

the methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable

Availability of data and materials

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

Competing Interests

All authors have no conflicts of interest that could be perceived to bias their work, making known all financial support and any other personal connections.

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Authors' contributions

C.H.W., H.L. designed the study, analyzed the data and helped write the manuscript. J.L. performed the immunohistochemical experiments and analyzed the data. C.H.W., H.C.F. performed statistical analysis. C.H.W. H.C.F. was involved in performing the experiments. Y.C.O., C.H.W., H.L., and H.C.F. provided the clinical resources and executed of the study. C.H.W., H.L. supervised the study and drafted the manuscript. All authors have reviewed and approved the final manuscript.

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Figures

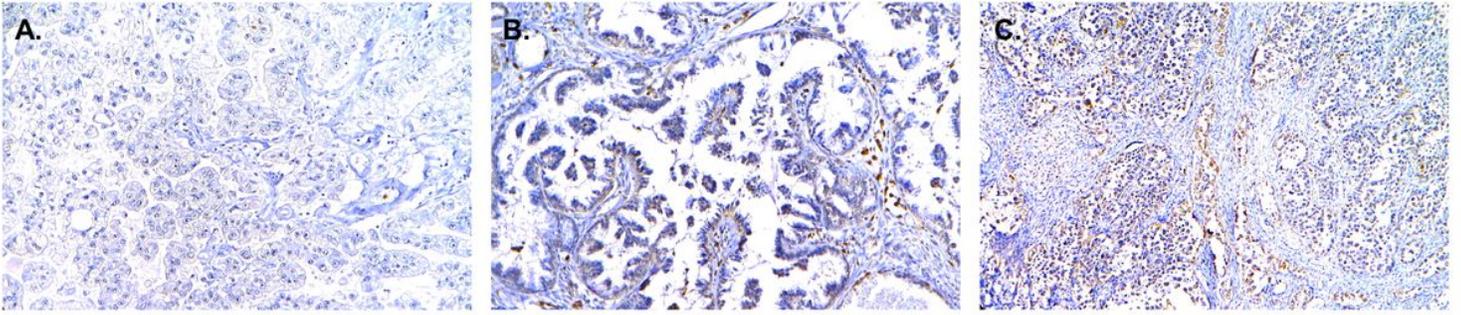


Figure 1

Representative cases of immunohistochemical staining for PR protein in ovarian clear cell carcinoma. (A) Negative expression. (B) Weak expression in 15% of the tumor cells with an H-score of 15. (C) Moderate expression in 40% of the tumor cells with an H-score of 80.

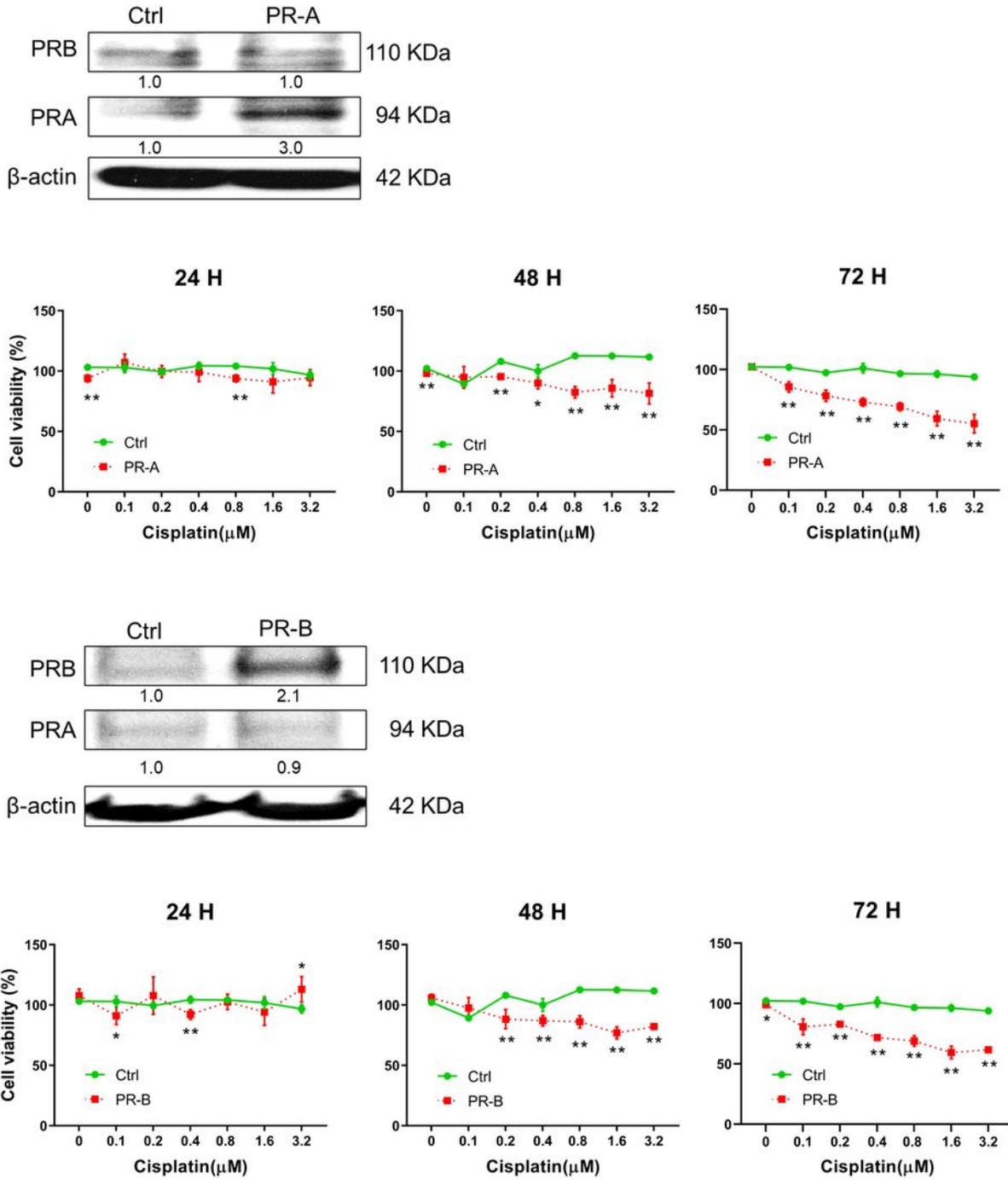


Figure 2

Western blot analysis of PR protein and comparison of cisplatin cytotoxicity in PR-overexpressed TOV-21G cancer cell lines. Experiments were performed in triplicate. Data was expressed as mean \pm SD of triplicate experiments. Control cells (green dotted line) were compared with PR-expressing cells (blue and red lines). * $p < 0.05$; ** $p < 0.01$. (A) Western blot analysis of PR-A protein levels and cisplatin cytotoxicity in

PR-A overexpressed TOV-21G cancer cell lines. (B) Western blot analysis of PR-B protein levels and cell viability assay in PR-B overexpressed TOV-21G cancer cell lines.