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ASSESSMENT OF LONG NON-CODING RNA LNC-RP11-697E AND ITS TARGET Hepatocyte Nuclear Factor 1 β AS PREDICTORS TO CISPLATIN- RESISTANCE IN OVARIAN CANCER

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ABSTRACT

The current study aimed to investigate the prediction utility of the long noncoding RNA (lnc-RP11_697E) and its target gene, hepatocyte nuclear factor 1β (HNF-1β) to Cisplatin-resistance in patients with ovarian cancer (OC), in addition, to assess their diagnostic significance. The expression levels of lnc-RP11-697E and HNF-1β were assessed in 25 formalin-fixed paraffin-embedded tissue (FFET) samples from OC patients who were treated with intravenous Cisplatin-based chemotherapy and 25 healthy ovarian (non-cancerous) tissues as the control group using qPCR. The results revealed that lnc-RP11-697E was significantly overexpressed, while HNF-1β was downregulated in Cisplatin-resistant tissues. In addition, lnc-RP11-697E and its target HNF-1β exhibited high sensitivity and specificity in predicting Cisplatin-resistance and in differentiating ovary cancer from controls. The tissue expression levels of lnc-RP11-697E and its target HNF-1β could be used as diagnostic biomarkers for OC and as predictors for Cisplatin-resistance in patients suffering from OC.

KEYWORDS: Ovarian cancer, HNF-1β, lnc RNAs, lnc-RP11-697E, Cisplatin resistance.

INTRODUCTION

Ovarian cancer (OC) is the fifth most prevalent cause of mortality and the most common cause of death among gynecological malignancies, with a survival rate of less than 30% within five-years.[1] Late diagnosis and a lack of appropriate treatment choices for resistant disease contribute to the high death rate of OC.[2] OC is currently treated by tumor cytoreductive surgery followed by adjuvant platinum-based chemotherapy which includes cisplatin.[3] Cisplatin arrests the cell cycle and interferes with DNA repair mechanisms by generating adducts with the purine bases on the DNA, inhibiting replication, and transcription, and ultimately leading to apoptosis in cancer cells.[4] cisplatin-based treatment is effective, but it has a significant negative impact on treatment outcomes due to drug resistance that represents the leading causes of increased mortality in patients with advanced cancer.[5] Multiple explanations have been proposed for the mechanisms of drug-resistance that can be developed in ovarian malignant tumor cells, that include decreased drug concentration inside the tumor cells, increased detoxification, increased DNA repair, apoptotic dysregulation, alterations in the tumor microenvironment, and evasion of the host immune response.[6], [7], [8], [9], [10]

Long non-coding RNAs (lncRNAs) are transcripts that are composed of more than 200 nucleotides with no protein-coding potentiality and have been identified to be expressed exclusively in some differentiated tissues and cancer types.[11] LncRNAs have numerous roles in gene regulation, nuclear domain organization and cis or trans transcriptional regulation.[12] lncRNAs have also been demonstrated to control carcinogenesis, invasion, metastasis, and cancer therapy resistance.[13], [14]

The microarray assay showed that are some lncRNAs that were dysregulated in cisplatin-resistant ovarian cancer tissues as contrasted with cisplatin-sensitive tissues.[15],[16] The data imply that the resistance behavior of cisplatin-resistant in patients with ovarian cancer is probably related to these differentially expressed lncRNAs, including the lnc-RP11_697E, which may be used as a potential biomarker for diagnosis or as a therapeutic target for cisplatin-resistant ovarian cancer.[17] lnc-RP11-697E is a long noncoding RNA that targets the gene Hepatocyte Nuclear Factor 1 (HNF-1β).[17] HNF1 β is a transcription factor that has a crucial role in the development and differentiation of numerous organs, including the liver, kidney, lung, gonads, biliary system, and pancreas.[18] The HNF-1β protein affects the

expression levels of many genes that are involved in cell cycle regulation, apoptosis susceptibility, and oxidative stress response.[19], [20]

This study aims to examine the predictive utility of the lnc-RP11_697E and its target gene HNF-1β for Cisplatin-resistance in OC patients by correlating their expression levels with the response to Cisplatin therapy, as well as to evaluate their diagnostic value in OC.

2. MATERIALS AND METHODS

2.1. Study Population

This study included 25 patients with OC from Ain Shams Internal Medicine Hospital's oncology department and 25 healthy ovarian (non-cancerous) tissues as the control group. Formalin-fixed paraffin-embedded tissue (FFET) from OC patients who are eligible for the study, as well as healthy ovarian tissue from age-matched women (patients aged >25 years old), was collected from patients undergoing adenexectomy for uterine prolapse or myoma. Patients with OC were given three to four cycles of intravenous platinum-based chemotherapy in the form of cisplatin and were not given any other chemotherapy.

Fifty ovarian tissue samples that were embedded in formalin-fixed paraffin-embedded (FFPE) were gathered and examined. Depending on how well they responded to the standard treatment plan of cisplatin-based chemotherapy, patients with OC were subdivided into two subgroups: the cisplatin-sensitive subgroup (n = 16) and the cisplatin-resistant subgroup (n = 9). Twenty-five patients with benign ovarian lesions who were not diagnosed as cancer patients made up the control group.

The protocol of this study was approved by the Institutional Review Board (IRB) Ethics Committee of Ain shams University prior to patient recruitment. Furthermore, this study was carried out in compliance with the World Medical Association's Helsinki Declaration.[21] Prior to the study, we received written informed consent from all subjects included in this study and told them that their information, as well as their data and medical records, would be kept confidential. At the time of enrollment, each patient was given a unique identifying number.

2.2. Total RNA extraction and reverse transcription from FFPE tissue blocks

The deparaffinization solution (Cat. No. 19093) was used to deparaffinize FFPE tissues. The total RNA was then extracted using the RNeasy FFPE Kit (Cat. No. 73504). The technique was carried out in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). The purity of the extracted RNA was determined by calculating the ratio of the optical densities that were measured at wavelengths 260 and 280 nm using an ultraviolet (UV) spectrophotometer (Eppendorf, Germany). In a total volume of 20 µl, total RNA was reverse transcribed using the QuantiTect RT kit (Cat. No. 205311) in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). The reaction mixture was adjusted at 37 °C for 60 min, then at 95 °C for 5 min to inactivate Quantiscript Reverse Transcriptase enzyme. Undiluted reaction mixtures were then transferred to a -20 ºC freezer to be stored till applying real time qPCR.

2.3. Gene expression analysis by Quantitative real-time PCR (qPCR)

The cDNA was amplified using primers sequences that are specific for the lnc-RP11-697E and HNF-1β. The used primer sequences are [For Human lnc-PR11-697E22.2; ID: LPH25763A; RT2 lncRNA primer assays cat no: 330701] and [For Human HNF-1β; ID: QT00034237; QuantiTect Primer Assay, cat no: 249900] (Qiagen, Hilden, Germany). The gene expression was normalized using primer sequence Hs_ACTB_1_SG QuantiTect Primer Assay, cat no: 249900, ID: QT00095431 for β-actin housekeeping gene. Quantification of cDNA targets was carried out using RT² SYBR Green qPCR Master-mix (Cat. No. 330504) for lncRNA and QuantiFast SYBR Green PCR Kit (Cat. No. 204056) for HNF-1β and housekeeping gene. The reaction mixtures and cycling protocols were adjusted using the 5 Plex Rotor-Gene PCR Analyzer (Qiagen, Hilden, Germany) in accordance with the manufacturer's instruction. The level of housekeeping gene expression was utilized to do data normalization for all markers. The relative quantification (RQ) was calculated using the $2^{-\Delta\Delta Ct}$ method and the data were presented as fold change (FC) of gene expression.

2.4. Statistical analysis

For normally distributed continuous variables, the data were presented as mean ± standard deviation, and as the median and interquartile range (IQR) for non- normally distributed variables. Frequencies and percentages were used to express categorical variables. For non-parametric comparisons between the groups, we employed the Mann Whitney U test. The median level of gene expression was used to derive the cut-off values. The specificity and sensitivity of biomarkers were assessed using receiver operator (ROC) curves. The Statistical Package for Social Science (SPSS-IBM, Version 23) was used for all statistical analyses. A p-value of < 0.05 was deemed the statistical significance cut-off point.

3. RESULTS

3.1. Demographic characteristics of the studied groups

3.1.1. Age

This current study included 50 individuals that were divided into two groups; group I (25) OC patients who received cisplatin chemotherapy, this group was then subdivided into two subgroups cisplatin resistant (9) and cisplatin sensitive (16) their ages ranged between 27 – 66 years with mean age of 49.6±11.0. Group II (25) patients from age-matched women with benign tumor and their ages ranged between 36 -62 years with mean age of 47.2±8.1. There was no significant difference observed

between the two groups (p-value>0.05), while there was a significant difference in the percentage of patients age (<50 vs >50) years in OC patients compared to patients with benign tumors (p=0.005) (Table 1).

3.1.2. Comorbidity

According to data in table 1 about 48% of patients with ovarian cancer had Diabetes Mellitus (DM), 52% had not. Patients with Benign tumors had Diabetes Mellitus (40%) and 60% did not, this means that there is no significant difference between the two groups in relation to DM (p-value > 0.05). Regarding hypertension, 84% of ovarian cancer patients did not show hypertension while 16% had hypertension. For Benign tumors patients, 60% did not have hypertension while 40 % had hypertension with no significant difference between the two groups (p-value > 0.05).

3.1.3. Clinico-pathological Characteristics of OC groups

In Cisplatin sensitive group patients, 14% with Adenocarcinoma,14% with serous type and 21% Mucinous, 64% with Endometroid carcinoma which is the high proportion rate between all Cisplatin Sensitive patients, While in Cisplatin resistant group, no one with Adenocarcinoma, 33% with serous, 11% with Mucinous and 55% with Endometroid carcinoma. The data revealed no statistically significant difference between the two groups (Table 2).

Grade I-II (84%), Grade III- IV (16%) in Cisplatin Sensitive and Grade I-II (66%), Grade III- IV (33%) in Cisplatin Sensitive group. There is no significant difference (p-value=0.11) between the two groups regarding histopathological grade. Regarding metastasis, there is no significant difference between Cisplatin Sensitive and Cisplatin resistant (p-value=0.92). (Table 2).

3.2. Tissue expression levels of lnc-RP11-697E and HNF-1β among the studied groups

The results showed that there was a significant increase in the levels of lnc-RP11-697E expression (Fig. 1a) while, the expression of HNF-1β gene (Fig. 1b) was downregulated in cases of OC compared to Benign tumor controls (p < 0.01) (Table 3).

On comparing the median values of lnc-PR11-697 and HNF1b expressions among the Cisplatin-sensitive and Cisplatin-resistant subgroups (Table 4), a significant increase in the expression levels of lnc-PR11-697 in resistant cases compared to sensitive cases was noted (Median = 10.43 vs 5.028; p-value = 0.0001) (Fig. 2a).On the other hand, a lower median expression levels of the HNF1b (Fig. 2b) was significantly associated with Cisplatin resistant ovarian tissues (p-value < 0.05).

3.3. Prediction potential of lnc-RP11-697 and HNF1b genes for resistance to Cisplatin therapy in OC patients

Based on our analysis, we found that lnc-RP11-697 and HNF1b genes can be used as accurate predictors for the resistance to Cisplatin-based treatment (Fig. 3). The accurate cut-off value was determined based on Receiving operator characteristics (ROC) curve, which was > 8.85 for lnc-RP11-697 and < 0.124 for HNF1b. The sensitivities and specificities of the calculated biomarkers were [(For lnc-RP11-697: 80% and 86.7%, p < 0.002), and (For HNF1b: 70% and 80%, p < 0.03)]; respectively (Table 5).

3.4. Diagnostic potential of lnc-RP11-697 and HNF1b genes for OC

In order to assess the diagnostic value of lnc-RP1-697 and HNF1b in OC; a ROC analysis revealed significant diagnostic potential for lnc-RP11-697 and HNF1b to discriminate ovary cancer from controls (p-value = 0.0001) (Fig. 4 and Table 6).

Table 1: Distribution of age (years) and comorbidities among the studied groups.

Group	parameter	Cancer ovary (n=25)	Benign tumors (n=25)	Statistics
Age (years)	mean ± SD	49.6±11.0	47.2±8.1	t=0.86, p-value: 0.39 (NS)
	Range	27.0 – 66.0	36.0 – 62.0	
Age group				
<50 (n=27)	N (%)	7(28%)	18 (72%)	P-value= 0.005 (HS)
>50 (n=23)		2 (20%)	20 (80%)	
		Diabetes Mellitus		
No (n=28)	N (%)	13(52%)	15 (60%)	P-value= 0.77 (NS)
Yes (n=22)		12 (48%)	10 (40%)	
		Hypertension		
No (n=36)	N (%)	21 (84%)	15 (60%)	P-value= 0.11 (NS)
Yes (n=14)		4 (16)	10 (40%)	

t: value of independent t-test, NS: no significant difference, SD: standard deviation, HS: High significant difference.

Table 2: Clinical Characteristics of the OC Group.

Group	parameter	Cisplatin Sensitive (n=16)	Cisplatin resistant (n=9)	Statistics
		Pathological group		
Adenocarcinoma	N (%)	2 (14%)	0 (0%)	P-value= 0.63 (NS)
Serous		2 (14%)	3 (33%)	

Mucinous		3 (21%)	1 (11%)	
Endometroid		9 (64)	5 (55%)	
Histopathological grade				
Grade I-II	N (%)	13 (84%)	6(66%)	P-value= 0.11 (NS)
Grade III- IV		3 (16%)	3 (33%)	
Metastases				
No	N (%)	12 (75%)	7 (77%)	P-value= 0.92 (NS)
Yes		4 (25%)	2 (23%)	

NS: no significant difference.

Table 3: Comparative analysis for the expression of lnc-RP11-697 and HNF-1β in OC tissue compared to benign tissue.

Group	parameter	Cancer ovary (n=25)	Benign tumors (n=25)	Statistics
Lnc-RP11-697	median	7.8	1.04	p-value: 0.0001 (HS)
	Range	2.0 – 19.0	0.62 – 4.08	
	75% percentile	9.78	1.75	
HNF-1β	median	0.087	1.117	p-value: 0.0001 (HS)
	Range	0.017 – 1.526	0.248 – 3.41	
	75% percentile	0.145	1.413	

HS: High significant difference

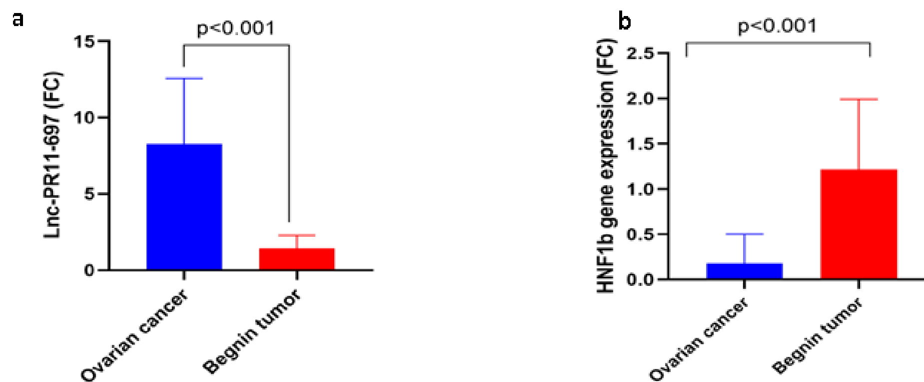

Fig. 1: Bar chart graphs present a significant difference in the expression levels of lnc-PR11-697 and HNF-1B in ovarian tumors versus benign tumors ($p < 0.01$). (a): higher expression levels of lnc-PR11-697 was detected in OC tissues, whereas; the HNF-1β (b) was downregulated, when compared with benign tissues.

Table 4: Comparative analysis for the expression of Lnc-RP11-697 and HNF-1B in Cisplatin-sensitive versus Cisplatin-resistant OC tissues.

Group	parameter	Cisplatin sensitive (n=16)	Cisplatin resistant (n=9)	Statistics
Lnc-RP11-697	median	5.028	10.43	p-value: 0.0001 (HS)
	Range	2.04 – 9.45	7.83 – 19.56	
	75% percentile	7.62	15.56	
HNF-1 β	median	0.144	0.057	p-value: 0.03 (S)
	Range	0.03 – 0.265	0.023 – 0.295	
	75% percentile	0.161	0.132	

S: significant difference, HS: High significant difference.

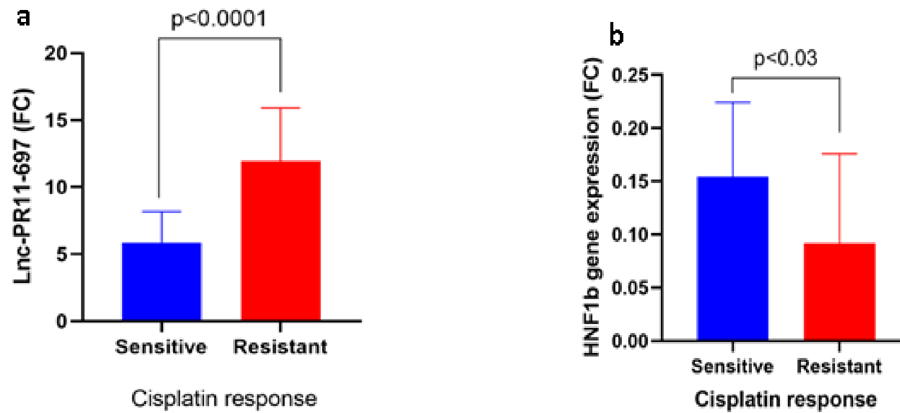

Fig. 2: Bar chart graphs present a significant difference in the expression of lnc-PR11-697 and HNF-1B in Cisplatin-sensitive and resistant OC patients (p-value < 0.01). (a): higher expression levels of lnc-PR11-697 was detected in resistant cases compared to sensitive ones, whereas; the HNF-1 (b) was downregulated in resistant cases, when compared with sensitive cases.

Table 5: Prediction potential of Lnc-RP11-697 and HNF-1B genes for resistance to Cisplatin therapy in OC patients (ROC Curve).

Parameter	AUC	95% CI	P value	Cut-off value	Sensitivity (%)	Specificity (%)
Lnc-RP1-697 (FC)	0.95	0.88 – 1.0	0.002	>8.85	80	86.7
HNF-1B (FC)	0.76	0.54 – 0.97	0.03	<0.124	70	80

AUC: Area under the curve; ROC: Receiving operator characteristics, CI: Confidence interval, FC: Fold change.

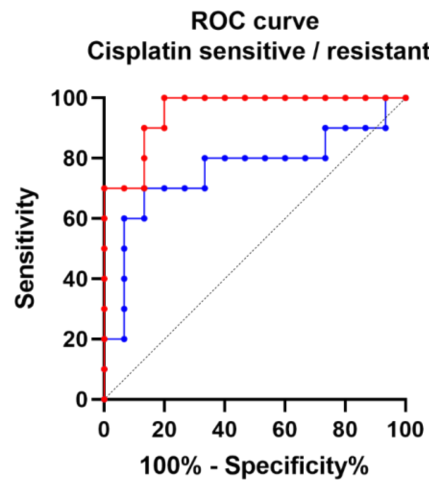

Fig. 3: ROC curve presents the predictive potential of lnc-RP11-697 and HNF-1B in OC.

Table 6: Diagnostic potential of Lnc-RP11-697 and HNF-1B genes in for Ovarian Cancer (ROC Curve).

Parameter	AUC	95% CI	P value	Cut-off value	Sensitivity (%)	Specificity (%)
Lnc-RP1-697 (FC)	0.98	0.96 – 1.0	0.0001	>2.65	96	88
HNF1b (FC)	0.93	0.85 – 1.0	0.0001	<0.48	88	80

AUC: Area under the curve; ROC: Receiving operator Characteristics, CI: Confidence interval, FC: Fold change.

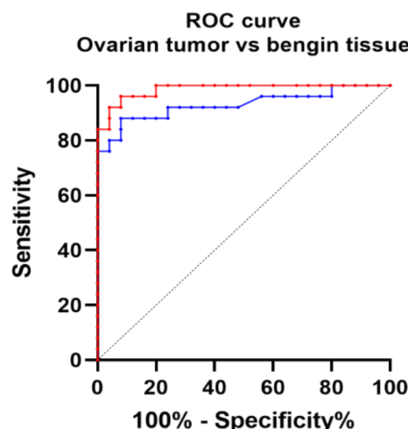

Fig. 4: ROC curve presents the diagnostic efficacy of lnc-RP11-697 and HNF1β in OC.

4. DISCUSSION

Despite the fact that cisplatin has been a pivotal chemotherapeutic agent in the treatment of different forms of cancer, including OC, drug resistance has been a serious clinical impediment. Mechanisms of drug resistance inside tumor cells may include lower drug accumulation, increased detoxification activity, promotion of DNA repair capacity, and inactivated cell apoptotic signaling.[22]

The current study was designed to investigate the potentiality of using the lnc-RNA "RP11-697E" and its target HNF-1β as diagnostic biomarkers for OC and as predictors to Cisplatin resistance in patients with OC. Up to date, no literatures have described the predictive and diagnostic potential of both lnc-RP11-697E and its target HNF-1β on clinical samples and all the previous studies were concerned to assess them experimentally by in vitro studies.

According to the results of the current study, the overexpression of lnc-RP11-697E and downregulation of HNF-1β in OC tissues compared to the healthy non-cancerous ones was significantly associated with Cisplatin-resistance. Furthermore, there was a significant diagnostic potentiality for lnc-RP11-697 and HNF1b to discriminate ovary cancer from controls.

HNF-1β is a homeobox transcription factor that is required for the expression of several genes throughout embryonic development and organ differentiation, primarily in the liver, kidney, and pancreas. Expression alteration and single nucleotide polymorphisms (SNPs) of HNF-1β gene have now been linked to a variety of tumors, including endometrial, prostate, ovarian, hepatic, renal, and colorectal cancers.[23] Epigenetic silencing of the HNF1b gene has also been found in certain human malignancies, including colorectal carcinoma, breast cancer, and OC.[24-26]

Kao et al.[27] was demonstrated that the overexpression of HNF1b is specific for ovarian CCC (clear cell carcinoma) among ovarian carcinomas which led to its use as diagnostic marker. The HNF1β gene is essential in the biology of ovarian CCC. HNF1β knockdown resulted in a significant increase in proliferation in ovarian CCC cells, whereas HNF1β overexpression resulted in a significant decrease in cell growth in the serous ovarian cancer cell line.[28] HNF1β downregulation may contribute to drug resistance in ovarian cancer, and HNF1β may conduct drug resistance-related functions via four pathways, including ErbB and p53 signaling, focal adhesion, and apoptotic pathway.[29]

The abnormal expression of HNF1β in tumours is linked to epigenetic processes and epigenetic alterations. Methylation of CpG island clusters is one of the epigenetic processes that regulates gene expression in humans. Hypermethylation of the HNF1β CpG island is one of the possible mechanisms for aberrant HNF1β downregulation in OC.[30] Histone acetylation and gene amplification are two of the potential causes of HNF1β overexpression that was observed in cells treated with a histone deacetylase inhibitor in combination with a methyltransferase inhibitor.[30]

Lnc-RP11-697E is a long intergenic non-coding RNA (lincRNA) that targets the gene HNF1β.[17, 31] LincRNAs are non-coding RNAs longer than 200 nucleotides in length and have well-defined functions in chromatin remodeling, RNA stability, and transcription regulation.[32] Different types of lincRNAs can regulate gene expression at different levels, from transcription to translation, either in cis (on neighboring genes) or in trans (on distant genes).[33-36, 12, 37] Intergenic and antisense lncRNAs have been shown to affect cell behavior in a range of cancers.[38-40]

Lnc-RP11_697E was associated with cisplatin-resistant ovarian cancer where it was markedly overexpressed in Cisplatin-resistant OC compared with the cisplatin-sensitive OC cells.[17] So, lnc-RP11-697E can be considered as an oncogene that has a negative effect on the response of ovarian cancer cell for treatment. The exact mechanism of how lnc-RP11-697E is involved in the regulation of drug- resistance in OC is unclear but

Gene Ontology (GO) analysis was performed to predict the potential function of this dysregulated lncRNA.[17] It has been demonstrated that lncRNA modulates a variety of biological processes, including the mitotic M phase, which are directly related to drug resistance in cancer cells.[41] Furthermore, pathway analysis revealed that this dysregulated lncRNA was involved in signaling pathways in humans, such as MAPK signaling, endocytosis, ubiquitin-mediated proteolysis, p53 signaling pathway, spliceosome, cell cycle, and oocyte meiosis, all of which have been extensively studied in OC initiation and progression.[41-43]

5. CONCLUSION

Based on our results, the tissue expression levels of lnc-RP11-697E and its target HNF-1β could be used as diagnostic biomarkers for OC and as predictors to Cisplatin-resistance in OC patients. Their accuracy as predictors for resistance and as diagnostic biomarkers for OC are significant. However, because of the small sample size in this study, more research is needed to comprehensively evaluate the clinical significance of these genes on a broad scale of OC samples.

2. Conflicts of interest

“There are no conflicts to declare”.

3. Formatting of funding sources

Nil.

4. ACKNOWLEDGMENTS

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