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Clinical significance of p27 ^{Kip1} expression in advanced ovarian cancer

Amani A. Alrehaili[1], Majed AlMourgi[2], Amal F Gharib[1,3], Wael H Elsawy[4*], Khadiga Ahmed Ismail[1,5], Howaida Mahmoud Hagag[1,6], Farah Anjum[1] and Nermin Raafat[3]

Abstract

Background: Ovarian cancer is the most common gynecological malignancy. In patients with advanced ovarian cancer, some biological parameters have prognostic implementations. P27^{kip1} is an inhibitor of a cycline-dependent kinase, its loss, can contribute to tumor progression.

Objective: This study aimed to examine the importance of P27^{KIP1} protein in predicting the prognosis and response to neoadjuvant chemotherapy in patients with advanced ovarian epithelial cancer and to compare the outcomes of immunohistochemistry with Quantitative Real-time PCR.

Patients and methods: We have studied P27^{KIP1}expression by both immunohistochemistry and Quantitative Real-time PCR from 88 patients with advanced ovarian carcinomas undergone radical debulking surgery and received Paclitaxel followed by Cisplatin every 3 weeks for a total of 6 cycles. We also studied their association with both chemotherapy response and patient survival.

Results: Nuclear expression of p27^{KIP1} protein was intense in 86 normal ovarian tissues and 42 of 88 carcinomas. The P27^{kip1}mRNA expression level by qRT-PCR was very low in ovarian cancer tissues relative to its adjacent normal tissues. The results were statistically significant by both methods of determination. p27^{KIP1} expression was significantly related to good prognostic parameters as low stage tumors, differentiated tumors, absence of ascites, residual disease < 2 cm, and response to chemotherapy but not with histopathological type in case of determination by immunohistochemistry. Comparison of P27^{kip1} by both immunohistochemistry and qRT-PCR with different prognostic parameters revealed no significant difference between both methods in the assessment of these parameters. In 4 years of follow-up, 20.5% of patients were alive without evidence of disease. 6.8% were alive with disease. The disease-related four -year survival rate for the whole group was 28.2%. In multivariate analysis, residual disease, histological type, tumor differentiation, ascites was of independent prognostic significance.

Conclusion: In ovarian cancer, patients with loss of p27KIP1 expression are at a greater likelihood of disease progression, p27^{KIP1} may be used as a molecular marker to predict response to chemotherapy and prognosis. Both immunohistochemistry and qRT-PCR have equal reliability in the determination of p27 ^{KIP1}.

Keywords: Ovarian cancer, p27 ^{Kip1}, Immunohistochemistry, RT-PCR, Chemotherapy

* Correspondence: whelsawy@gmail.com
[4]Clinical Oncology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt
Full list of author information is available at the end of the article

Introduction

Epithelial ovarian cancer is the most fatal gynecological malignancy in developing countries [1]. Approximately 80% of patients are diagnosed with an advanced stage [2], which is associated with a 5-year survival rate of only 30%. This, despite improvements in long-term survival, obtained through the use of combination chemotherapy, mainly with cisplatin and lately with paclitaxel [3]. Several factors attribute to the bad prognosis of patients with an advanced stage ovarian carcinoma, including the inability to perform complete cytoreductive surgery to eradicate the metastatic disease in > 75% of patients, underlying resistant to chemotherapy in over half of these patients and the emergence of chemoresistance in approximately half of the originally sensitive patients [4]. Although clinical-pathological characteristics of ovarian cancer other than the stage of the disease, such as residual disease size following debulking surgery, histological grade and type, lymph node status and the existence of ascites are additionally of proven prognostic value, individual patients may show considerable chemosensitivity variations although they share the same clinical-pathological characteristics [5]. The pathogenesis of most human cancers involved dysregulation of normal cell cycle control. Abnormal expressions of regulatory proteins that regulate G1-S phase transition are frequently noticed as a critical, rate-limiting step in the progression of the cell cycle. G1-S transformation involves phosphorylation of the retinoblastoma protein pRb, leading to the production of transcription factors in the E2F family, which activate genes necessary for entry into the S phase [6]. PRb phosphorylation is launched by complexes of cyclin D1/(CDK)4–6 and ended in late G1 by cyclin E/CDK2. Changes in the expression of cyclin and/or cycline-dependent kinase (CDK) lead to enhanced cell proliferation and are undoubtedly believed to contribute to malignancy. Also, down-regulation or inactivation of CDK inhibitors, including p21Waf1/Cip1, $p27^{Kip1}$, and p16Ink4a, usually causing G1 arrest by binding to cyclin-CDK complexes, is frequently found in various human tumors, making sufficiently the cell vulnerable to uncontrolled extracellular proliferation signals further [7]. Several trials have found expression in epithelial ovarian cancer (EOC) of these critical cell cycle regulatory proteins. Despite frequent detection of alterations in expression levels, there are many conflicting results, making it hard to delineate the role of individual genes in the development and progression of EOC [8]. Several trials have shown that $p27^{KIP1}$ is a promising molecular marker of the poor prognosis in several cancers [9–13]. Several trials have detected an expression in epithelial ovarian cancer (EOC) of this critical cell cycle regulatory protein. Despite frequent detection of alterations in expression levels, there are many conflicting results, making it hard to carefully delineate the role of particular genes in the potential development and progression of EOC [8]. Several trials have sufficiently shown that $p27^{KIP1}$ is properly a promising molecular marker of the poor prognosis in several cancers [9–13].

Patients and methods

Patients

This study comprises 88 patients with the International Gynecology and Obstetrics Federation (FIGO) [14] stage III epithelial cancer ovary confirmed histopathologically between May 2012 and April 2019. All patients underwent initial debulking surgery within 6 weeks before chemotherapy. Written informed consent has been obtained from all cases included. The minimum age for active recruitment in the study was at least 18 years of age. All patients had a normal blood picture, renal and hepatic function at the entry of the study. The study was approved by the Committee of Ethics of research, Zagazig University. Informed consent was obtained from all participating patients before enrollment in the study.

Exclusion criteria included patients with ovarian low malignant potential tumors; poor performance status of over 2 Eastern Cooperative Oncology Groups (ECOG) [15]; estimated glomerular filtration rate (GFR) of less than 60 mL/minute; severe neuropathy; prior chemotherapy or radiotherapy for ovarian cancer and congestive heart failure or arrhythmias.

Management

Postoperative chemotherapy comprised Paclitaxel 175 mg/m2 IV over 3 h followed by Cisplatin 75 mg/m2 IV infusion after vigorous hydration was typically administered every 3 weeks for 6 cycles for all patients included in the study. Gynecological examination, abdominopelvic ultrasonography, CA-125 assay were performed monthly. Other radiological studies, such as CT or MRI of the abdomen and pelvis, were performed before chemotherapy and every two-month if typically needed for Clinical response evaluation according to the revised RECIST (Response Evaluation Criteria in Solid Tumors) criteria [16].

Four weeks after the 6th cycle of chemotherapy, patients with a clinical complete response underwent a laparoscopy. In laparoscopy negative cases, a laparotomy was performed to evaluate the pathological response by multiple biopsies. After pathological assessment of biopsies, patients were properly assigned to one of three groups: complete response, partial response with the microscopic disease only, and persistent disease.

Immunohistochemistry

Immunohistochemistry was performed as mentioned previously [17]. Briefly, Six-micron sections were cut from formalin-fixed paraffin-embedded tissue blocks,

deparaffinized in xylene, and rehydrated. For antigen retrieval and detection of $p27^{KIP1}$, the sections were heated in a microwave oven for a total of 30 min (three cycles of 10 min each) in 10 mmol/L sodium citrate buffer at pH 6.0. Endogenous peroxidase activity was eliminated by preincubation in 2% H2O2 in methanol for 30 min followed by three washes in phosphate-buffered saline (PBS). The sections were stained using standard streptavidin-biotin complex immunoperoxidase methods (Santa Cruz Biotechnology, Santa Cruz, CA) on a Ventana ES machine (Ventana Medical Systems, Tucson, AZ). The primary antibodies for $p27^{KIP1}$ were NCL-P27 monoclonal antibody (1:40). All antibodies were diluted in PBS containing 1% normal rabbit serum. Peroxidase activity was localized with chromogen 3,3′-diaminobenzidine tetrachloride in 0.5 mmol/L Tris buffer. The slides were counterstained with Delafield's hematoxylin. Normal ovarian tissue served as a positive control for $p27^{KIP1}$ immunostaining.

$p27^{Kip1}$ scoring

Samples were coded and the percentage of immunostained cells was assessed. Expression was categorized as positive (staining in ≥5% of cells) or negative (staining in < 5% of cells) as described previously. At least 20 high-power fields were selected randomly and 2000 cells were counted [18].

Quantitative real-time polymerase chain reaction

RNA extraction

RNA extraction The extraction of total RNA from tissue samples (normal and cancerous ovarian tissue) was performed using the RNase Kit (Qiagen, Germany). The purity and concentration of RNA were verified by evaluating the optical density (OD) at 260 and 280 nm using a spectrophotometer with an acceptable A260/A280 ratio of 1.8 to 2.1.

Quantitative real time-PCR of $P27^{kip1}$ mRNA gene expression

One microgram of RNA was transcribed reversely using the (QuantiTect Reverse Transcription Kit) as instructed by the manufacturer. P27kip1 expression was evaluated by quantitative real-time PCR (qRT-PCR) using the following primers: P27 kip1 forward primer: GGCTTTCAGATTCCCAACTT and P27 kip1 reverse primer: AGCCTCCCCACTCTCGTCT and ABL forward primer: AGTCTCAGGATGCAGGTGCT and ABL reverse primer: TAGGCTGGGGCTTT TTGTAA as ABL has been regarded to be a housekeeping gene.

PCR amplification was conducted in 25 μl of 12.5 μl 2x QuantiFast SYBR Green PCR Master Mix, 1 μM of each primer and 2 μl of cDNA under the following circumstances:

Thermal cycling conditions for each reaction included an initial hold at ninety-five °C for ten minutes, followed by forty denaturation cycles at ninety-five °C for ten seconds and annealing/extension at sixty °C for thirty seconds. Fold change in the expression of mRNA of cancerous and normal control tissue samples was obtained using the Livak method. $P27^{kip1}$expression level was determined by Stratagene, MX3000P Quantitative PCR System (Agilent Technologies), and evaluated using MxPro QPCR Software (Agilent Technologies). The kit was provided by QIAGEN, Valencia, CA, USA. The $2^{-\Delta\Delta CT}$ method [19] was used to calculate the relative level of gene expression compared to the β-actin housekeeping control.

Statistical analysis

Analysis of the data was implemented using the SPSS Statistics 16.0 (SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 6.07 software (La Jolla, CA, USA).

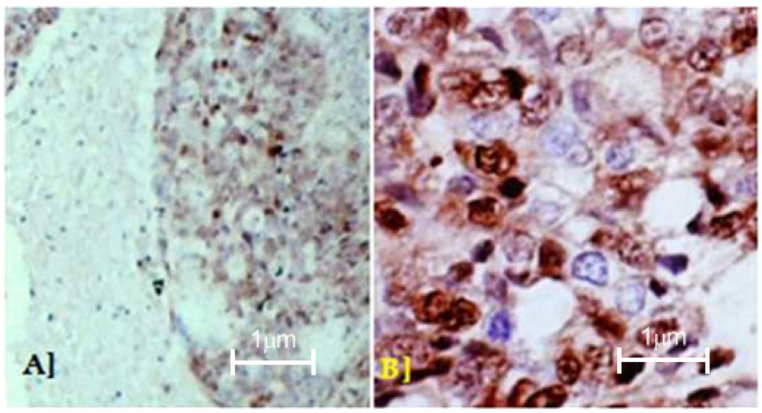

Fig. 1 Histological sections showing $p27^{KIP1}$ immunostaining in serous carcinoma representative of a high expression shows > 50% nuclear reactivity for $p27^{KIP1}$ in malignant epithelial cells. **a** ×100 and **b** × 400, Scale bar: 100 μm

Results

P27^{kip1} expression

P27^{kip1} has been analyzed using immunohistochemistry and quantitative real-time PCR. Immunoreactivity for P27^{KIP1} antigens has shown nuclear staining and weak cytoplasmic staining. Nuclear expression of p27^{KIP1} protein was intense in 86 normal adjacent ovarian tissues (NAOT) and in 42 of 88 carcinomas (ECO) (47.7%), the difference between both groups was statistically significant (t = 6.811, $p <$ 0.001) (Fig. 1 and Fig. 2 a).

The P27^{kip1}mRNA expression level by qRT-PCR in malignant ovarian tissues ranged between 0.001and 1.914 with a mean ± SD level of 0.743 ± 0.54 while in the normal adjacent ovarian tissues, the mRNA ranged between 5.6 and 15.35 with a mean ± SD level of 10.098 ± 2.716. The difference between both groups was statistically significant (t = 31.682, $p <$ 0.001) (Fig. 2b).

P27^{kip1}, clinical, and pathological characteristics

In the present study, the mean age of the patients was 44 ± 10.3 years (range 21–66 years). Sixty-six (75%) patients had an initial stage IIIC; 58 (65.9%) had moderate or poorly differentiated tumors. Serous carcinomas were the most prevalent pathological type (75%) and ascites were present in 60 (68.2%) patients. After initial debulking surgery, 56 patients (63.6%) had a residual disease greater

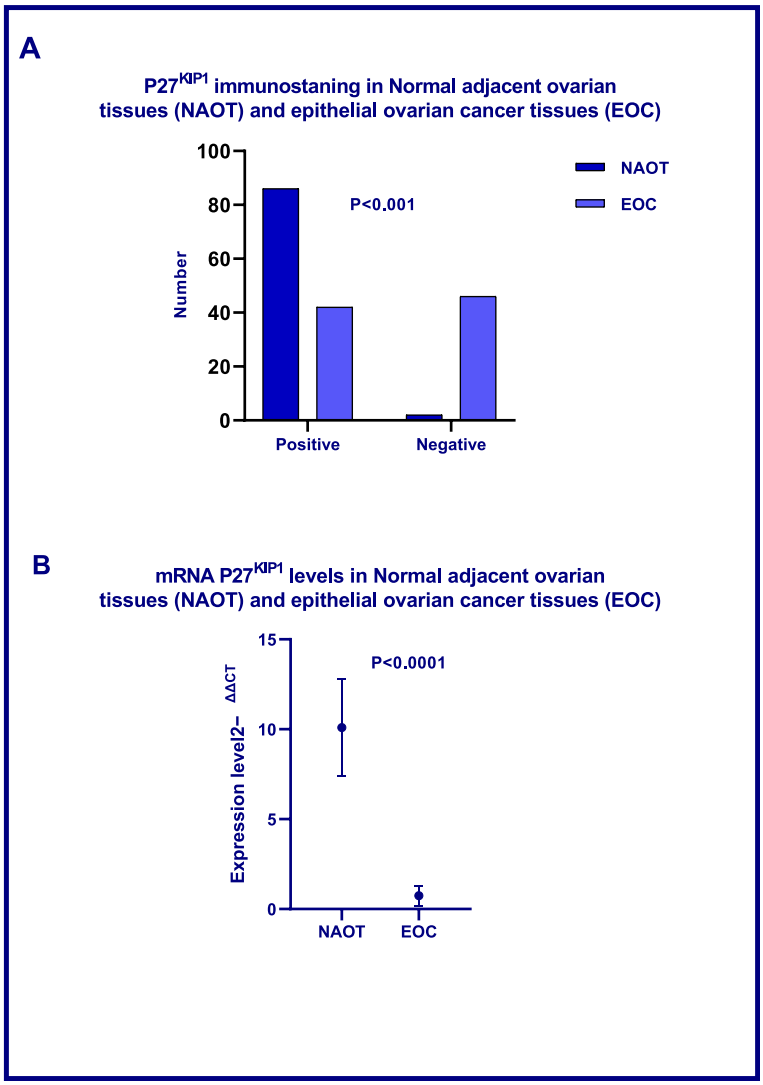

Fig. 2 P27 kip1 expressions in ovarian cancer tissues and their normal adjacent ovarian tissues. **a** Number of patients with P27^{KIP1} positive and negative immunostaining ($P < 0.001$). **b** Epression levels of mRNA P27^{KIP1} in ovarian cancer tissues and their adjacent normal tissues by RT-PCR ($P < 0.0001$)

than 2 cm in size. After chemotherapy, forty-six patients (52.3%) achieved a treatment response (Table 1).

$P27^{kip1}$was studied with the clinical and pathological data of the patients. According to $P27^{kip1}$ immunoreactivity, we observed a statistically significant relation between $P27^{kip1}$ expression and non-expression by immunohistochemistry and FIGO stage, tumor grade, the presence or absence of ascites, residual tumors after surgery and response to chemotherapy. No difference was observed between the serous and non-serous pathological types (Table 1). When analyzing the clinical and pathological data with $P27^{kip1}$ upregulation and downregulation by qRT-PCR, we found a statistically significant difference between all clinical and pathological data and up or down P27kip1 regulation (Table 1).

$P27^{kip1}$ and prognostic parameters

Expression of $P27^{kip1}$by immunohistochemistry was associated with good prognostic parameters as low stage tumors ($P = 0.040$), differentiated tumors ($P < 0.001$), absence of ascites ($P < 0.001$), residual disease < 2 cm ($P < 0.001$) and response to chemotherapy ($P = 0.004$) but was uncorrelatedwith the histopathological type (Table 1). Measurement of $P27^{kip1}$ mRNA by qRT-PCR revealed statistically significant upregulation with all prognostic parameters as analysis with immunohistochemistry but including the histopathological type (Table 1).

Statistical evaluation of the patients with positive P27 immunostaining and upregulation by RT-PCR with each of the clinical and pathological parameters revealed no significant difference between both techniques and all parameters (Table 2). Meanwhile, analysis of the patients according to their P27 expression or non-expression by IHC and according to up or downregulation by RT-PCR and each of clinical and pathological parameters revealed a significant correlation with each parameter (Table 2).

Comparison of $P27^{KIP1}$ expression by both immunohistochemistry and qRT-PCR with each subcategory of different prognostic parameters revealed no significant difference between both methods in the assessment of these parameters (Table 3).

$P27^{KIP1}$ and patients survival

During 4 years of follow-up, 18 patients (20.5%) were alive without evidence of disease; six were alive with disease (6.8%), whereas 64 had died of ovarian cancer (72.7%). The disease-related 4-year survival rate for the whole group was 28.2%. The median progression-

Table 1 Relation of expression of $P27^{KIP1}$ by Immunohistochemistry and RT-PCR and clinical and pathological data

	IHC						mRNA P27					
	P27 + ve		P27 -ve				Upregulation of P27		Downregulation of P27			
	N	%	N	%	t-test	P	N	%	N	%	t-test	P
Stage												
IIIA[a]	8	80	2	20	2.4	0.04	8	80	2	20	2.372	0.042
IIIB[a]	10	83.3	2	16.7	3.09	0.01	10	83.3	2	16.7	3.093	0.01
IIIC[a]	24	36.3	42	63.7	2.32	0.02	24	36.3	42	63.7	2.315	0.024
Grade												
Moderate + Well differentiated	26	86.7	4	13.3	5.92	< 0.001	26	86.7	4	13.3	5.92	< 0.001
Poorly differentiated	16	27.6	42	72.4	3.82	< 0.001	16	27.6	42	72.4	3.816	< 0.001
Pathology												
Serous	38	61.3	24	38.7	1.83	0.07	40	62.5	24	37.5	2.066	0.043
Non- serous	4	15.4	22	84.6	4.89	< 0.001	2	8.3	22	91.7	7.405	< 0.001
Ascites												
Absent	24	85.7	4	14.3	5.92	< 0.001	24	85.7	4	14.3	5.396	< 0.001
Present	18	30	42	70	3.38	0.00	14	23.3	46	76.7	4.892	< 0.001
Response												
CR, Microscopic disease, PR[a]	40	87	6	13	7.46	< 0.001	41	89.1	5	10.9	8.509	< 0.001
No response or progressive disease	2	4.8	40	95.2	13.7	< 0.001	1	3.4	41	97.6	17.43	< 0.001
Residual Disease												
> 2 cm	18	30	42	70	3.38	0.00	15	25	45	75	4.472	< 0.001
< 2 cm	24	85.7	4	14.3	5.4	< 0.001	21	75	7	25	3.055	0.005

[a]*CR* Complete response, *PR* Partial response

Table 2 *P* values of statistical analysis of different clinical and pathological parameters

	Mann Whitney U test[a]	Pearson's X2[b]
Tumor stage	> 0.9999	< 0.0001
Histopathological Grade	> 0.9999	< 0.0001
Pathological type	> 0.9999	< 0.0001
Ascites	> 0.9999	< 0.0001
Response to treatment	> 0.9999	< 0.0001
Residual disease	0.6667	< 0.0001

[a]Mann Whitney U test between Positive p27 by IHC and increased expression by RT-PCR and different parameters, [b]Pearson's X^2 test between positive and negative P27 by IHC and by RT-PCR and different parameters

free survival time was 11 months, and the median corrected survival time was 21 months. In univariate analysis, FIGO stage ($P = 0.04$), histological type ($P = 0.094$), differentiation grade ($P < 0.001$), ascites ($P < 0.001$), residual disease ($P < 0.001$) and response to chemotherapy ($P = 0.004$) were correlated to corrected survival. Expression p27 ^{KIP1} by immunostaining or qRT-PCR did not reach statistical significance when its effect on survival was examined. Meanwhile, in multivariate analysis, residual disease, histological type, differentiation, ascites was of independent prognostic significance (Table 4).

Table 3 Comparison of P27kip1 assessment by immunohistochemistry, qRT-PCR, and different prognostic parameters

	No.		RNA		IHC		X^2	P
Stage			No	%	No	%		
IIIA	10	Upreg[a] or positive IHC[b]	8	80	8	80	0.313	0.576
		Downreg.[c] or negative IHC	2	20	2	20		
IIIB	12	Upreg or positive IHC	10	83.3	10	83.3	0.3	0.584
		Downreg. or negative IHC	2	16.7	2	16.7		
IIIC	66	Upreg or positive IHC	42	63.6	42	63.6	0.033	0.856
		Downreg. or negative IHC	24	36.4	24	36.4		
Grade								
Mod. + Well[d]	30	Upreg or positive IHC	26	86.7	26	86.7	0.144	0.704
		Downreg. or negative IHC	4	13.3	4	13.3		
Poor	58	Upreg or positive IHC	16	27.6	16	27.6	0.043	0.835
		Downreg. or negative IHC	42	72.4	42	72.4		
Pathology								
serous	64	Upreg or positive IHC	43	67.2	38	59.4	0.538	0.463
		Downreg. or negative IHC	21	32.8	26	40.6		
NS[e]	24	Upreg or positive IHC	2	8.3	4	16.7	0.19	0.663
		Downreg. or negative IHC	22	91.7	20	83.3		
Ascites								
present	60	Upreg or positive IHC	14	23.3	18	30	0.384	0.536
		Downreg. or negative IHC	46	76.7	42	70		
absent	28	Upreg or positive IHC	24	85.7	24	85.7	0.146	0.703
		Downreg. or negative IHC	4	14.3	4	14.3		
Resid Dis[f]								
> 2 cm	60	Upreg or positive IHC	15	25	18	30	0.167	0.683
		Downreg. or negative IHC	45	75	42	70		
< 2 cm	28	Upreg or positive IHC	21	75	24	85.7	0.453	0.501
		Downreg. or negative IHC	7	25	4	14.3		
Response								
CR, Micro dis., PR[g]	46	Upreg or positive IHC	41	89.1	40	87	0	>.999
		Downreg. or negative IHC	5	10.9	6	13		
NR or progressive dis.[h]	42	Upreg or positive IHC	1	2.4	2	4.8	0	>.999
		Downreg. or negative IHC	41	97.6	40	95.2		

[a] Upregulation; [b] Immunohistochemistry; [c] downregulation; [d] Moderate and well-differentiated; [e] non-serous; [f] residual disease; [g] CR complete response, Micro dis.: microscopic disease, PR: partial response and [h] NR no response, progressive dis.: progressive disease

Table 4 Multivariate analysis of prognostic factors, using corrected survival as an endpoint

Variable	Grouping	*P*	Ratio of risk	95% CI
Response	CR, Micro dis., PR vs NR or progressive dis.	0.013	1.05	0.45–1.42
Residual disease	< 2 cm vs > 2 cm	0.0012	1.95	1.29–2.92
Grade	Well vs. moderate/poor	0.0034	2.69	1.34–5.37
Pathology	Serous vs. non-serous	0.043	1.42	0.99–2.04
Ascites	Present vs. absent	0.048	1.47	1.01–2.16

Discussion

In this study, the prognostic role of $p27^{kip1}$ expression was evaluated in 88 patients with locally advanced ovarian cancer. $P27^{kip1}$ was regarded as a tumor suppressor gene and the loss of its function was associated with the development of many kinds of human cancer. The tumor suppressor function of $p27^{kip1}$ was first involved in the regulation of the cell cycle [20]. Several trials have shown that expression of the CDK inhibitor p27 ^{KIP1} is a good predictor of longer time to progression and overall survival in many ovarian cancer patients [21–24]. Other studies evaluating the prognostic function of p27 ^{KIP1} expression in various types of tumors. In specific, loss of p27 KIP1 expression markedly improves the risk of recurrence and death related cancer in breast [25], prostate [26], bladder [27], hepatocellular [28], and colorectal [29] carcinomas. Although p27 expression appears to be an important predictor of clinical behavior in several malignancies, current evidence suggests that loss of $p27^{Kip1}$ protein is not attributable to structural alterations of the gene [20] but may result from increased degradation of the protein-mediated by ubiquitin-proteasome pathway [29, 30].

In the present study, correlations were observed with favorable prognostic factors such as lower FIGO stage, differentiated tumors, absence of ascites and residual disease < 2 cm and response to chemotherapy. p27 ^{KIP1} was shown not to be of prognostic significance in advanced ovarian cancer by both immunostainings and measurement of mRNA by real-time PCR. This is comparable with the results of Mudan Lu et al., that discovered that p27 ^{KIP1} expression by immunohistochemistry had independent prognostic significance in the meta-analysis, including nine trials: six conducted in Europe, three conducted in the USA, and one study conducted in Asia [31]. Hafez et al. [32] also discovered the same outcomes, by evaluating the concentrations of gene expression using real-time PCR and Western blotting.

We also examined the possible predictive value of p27 ^{KIP1} in the prediction of response to chemotherapy, as there is increasing evidence that p27 KIP1 functions as a regulator of drug resistance in solid tumors [30]. In human colon cancer cells, overexpression of $p27^{KIP1}$ was linked to increased resistance to drugs like cisplatin, doxorubicin, and etoposide [30], these drugs also used in the treatment of ovarian cancer. In our study, a significant correlation was found between the level of $p27^{KIP1}$ expression and response to chemotherapy, as its decreased expression was linked to no or poor response to chemotherapy. Mudan Lu et al. [31] in their meta-analysis also observed that $p27^{KIP1}$-positive cases have a higher response to chemotherapy, especially in patients who were optimally cytoreduced at first surgery. Platinum-based chemotherapy induces apoptosis in tumor cells, and reduced susceptibility to apoptosis has been proposed as a major mechanism responsible for chemotherapy resistance [33]. Recent data also suggest that $p27^{KIP1}$ overexpression induces apoptosis in many types of cancers through a p53-independent pathway [34–37]. Additionally, in many human breast cancer specimens, $p27^{KIP1}$ levels showed a significant correlation with the apoptotic index and predictive value for the benefit of chemotherapy [34].

$P27^{KIP1}$ expression may confer possible p53-independent apoptosis sensitivity, thus increasing the sensitivity of ovarian cancer cells to chemotherapy agents.

Conclusion

This study provides evidence about the role of p27 ^{KIP1} in ovarian cancer patients as a predictor for patient outcomes. Patients with ovarian cancer who have a loss of $p27^{KIP1}$ expression are at a greater likelihood of disease progression and may eventually benefit from more aggressive therapy. The reliability of p27 ^{KIP1} as a possible marker in the clinical routine evaluation and management of ovarian cancer merits further analysis in a study involving a large number of patients. Finally, this study provides evidence of equal reliability of immunohistochemistry and qRT-PCR in the determination of p27 ^{KIP1}.

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Authors' contributions

Amani A Alrehaili performed the research and analyzed the data. M AlMourgi: performed the research and analyzed the data. Amal F Gharib: designed the research study; performed the research; analyzed the data and wrote the paper. W H Elsawy: designed the research study; performed the research; analyzed the data and wrote the paper. Khadiga Ahmed Ismail: performed the research and analyzed the data. Howaida Mahmoud Hagag: performed the research, analyzed the data and contributed essential reagents. Farah Anjum: analyzed the data. Nermin Raafat: performed the

research, analyzed the data and contributed essential reagents. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Ethics approval and consent to participate
The study was approved by the Committee of Ethics of research, Zagazig University. Informed consent was obtained from all participating patients before enrollment in the study.

Consent for publication
All authors consent to the publication of the manuscript in ACR, should the article be accepted by the Editor-in-chief upon completion of the refereeing process.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Clinical Laboratory Department, College of Applied Medical Sciences, Taif University, Taif, Saudi Arabia. [2]Department of Surgery, Medical College, Taif University, Taif, Saudi Arabia. [3]Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt. [4]Clinical Oncology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt. [5]Medical Parasitology Department, Faculty of Medicine, Ain-Shams University, Cairo, Egypt. [6]Pathology Department, Al Azhar University Egypt, Cairo, Egypt.

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