

RESEARCH ARTICLE

MDM2 Expression in Serous and Mucinous Epithelial Tumours of the Ovary

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Abstract

Background: Different types of cancer exhibit abnormalities in cell cycle regulators. The murine double minute-2(MDM2) cell cycle regulator is a proto-oncogene that negatively regulates the P53 tumour suppressor gene. Surface epithelial tumours constitute approximately two thirds of ovarian neoplasms. Each histologic type can be classified as benign, borderline and malignant. This study aimed to examine immunohistochemical expression of the MDM2 protein in ovarian serous and mucinous epithelial tumours (benign, borderline and malignant). **Materials and Methods:** This study included forty five ovarian tumours, subdivided into fifteen cystadenomas (5 serous and 10 mucinous), fifteen borderline tumours (11 serous and 4 mucinous) and fifteen cystadenocarcinomas (9 serous and 6 mucinous). Paraffin sections were stained with haematoxylin and eosin for histopathologic study, and with mouse monoclonal anti-MDM2 antibody for immunohistochemistry. **Results:** MDM2 positivity was detected in 28.9% of the studied ovarian tumours. All benign tumours were negative and positivity was significantly higher in malignant than borderline tumours (P value of chi-square test =0.000). Significantly, all MDM2 positive mucinous tumours were malignant with no positive mucinous borderline tumours. Malignant tumours showed positive MDM2 expression in 83.3% of mucinous type and in 55.6% of serous type. Borderline serous tumours showed negative MDM2 in 72.7% of cases (P value of Z test =0.04). **Conclusions:** Alterations in the expression of the cell cycle regulator (MDM2) occur early in the process of tumourigenesis in serous and mucinous ovarian tumours. We suggest that MDM2 may be used in those tumours as a marker for risk stratification and identification of cases with cancer development and progression. We recommend further studies on MDM2 immunohistochemistry, in conjunction with adjuvant methods as DNA ploidy and FISH gene amplification, focusing on the mucinous tumours and differentiating between the three tumour categories, benign, borderline and malignant.

Keywords: Ovarian tumours - serous; mucinous - benign - borderline - malignant - MDM2

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Introduction

In Egypt, tumours of the female genital system represent 4.1% of total malignancies, ovarian cancer representing 1.37% of them. Surface epithelial tumours represent 73.33% of ovarian tumours; serous cystadenocarcinoma representing 34.82% and mucinous cystadenocarcinoma representing 17.04% of them (Mokhtar et al., 2007). In developed countries, ovarian cancer is the leading cause of death among gynecological malignant tumours. Most female patients present after disease has spread beyond the ovary. This causes high mortality inspite of treatment efforts (Khandakar et al., 2014). Therefore, early diagnosis of ovarian carcinoma is essential, to give chance for effective therapy (Ono et al., 2000).

Ovarian surface epithelial tumours are classified histopathologically into subtypes: serous, mucinous,

endometrioid, clear cell, transitional cell, squamous, mixed, and undifferentiated. Usually each subtype can be classified as benign, borderline, and malignant. Borderline tumour cases show excellent prognosis, while advanced cancer patients show five-year survival rates less than 25% (Lee, 2003).

Attention has been paid to understanding the types of ovarian carcinoma using light microscopy together with adjuvant methods as immunohistochemistry, and molecular studies. This may help in their differential diagnosis (Gilks and Prat, 2009). There is a progress in immunohistochemistry technique. Numerous antibodies to oncogenes and tumour suppressor genes are now available (Soussi et al., 2001).

The murine double minute-2 (MDM2) is a cell cycle regulator; negatively regulating P53 which is a tumour suppressor gene (Uhrinova et al., 2005). MDM2 is a

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proto-oncogene located on chromosome 12 (Dogan et al., 2005). MDM2 protein overexpression causes simulation to the mutant inactive P53 (Wang et al., 2001). Serous borderline tumours are negative for p53, whereas strong expression of this marker is observed in up to 50% of serous carcinomas (Kaspar and Crum, 2015).

MDM2 may be used as a marker for diagnosis (Rayburn et al., 2005), as a marker for advance in stage in bladder and prostatic carcinomas, non-Hodgkin's lymphomas and germ cell tumours of testis (Tuna et al., 2003), as a tumour progression marker in soft tissue sarcomas (Tuna et al., 2004) and as a prognostic marker in epithelial ovarian carcinoma (Dogan et al., 2005). Association between risk for ovarian cancer and MDM2 309 polymorphism has also been reported (Ma, 2013).

Normally, MDM2 protein binds with p53 gene in a complex. MDM2-p53 complexes in the nucleus are transported to the cytoplasm via signals present in the MDM2 protein, where p53 is degraded in the proteasome. Thus MDM2 acts as a nuclear-cytoplasmic shuttle for the p53 protein. P53 activates MDM2, which, in turn, down regulates p53. Following exposure to stress, the ability of MDM2 to bind to p53 is blocked preventing degradation caused by MDM2. Therefore, elevation of P53 occurs, resulting in arrest of the cell cycle (Manfredi, 2010).

After damage to DNA, p53 is phosphorylated at its N-terminus which harbours the domain necessary for binding MDM2. This negatively affects the p53- MDM2 interaction causing inhibition of the ability of MDM2 to target degradation of p53 by proteasomes. Ultimately, this leads to a elevation of p53. After the repair of DNA, MDM2 levels increase resulting in inhibition of p53 transcriptional activity and the degradation of p53 protein. The MDM2 protein has additional domains other than the one binding P53, possibly with additional functions. Thus MDM2 itself could be a target for cancer therapeutic intervention (Jenkins et al., 2012).

MDM2 protein affects the fate of other cell cycle regulators and thus affects the sensitivity of tumours to chemotherapy (Dolfi et al., 2014). Patients with wild-type TP53 high grade ovarian serous carcinomas appeared to have a poorer survival and were more chemoresistant than those with mutated TP53 (Wong et al., 2013). As MDM2 overexpression inactivates wild type p53 in several tumours; this may inspire us through new strategies for chemotherapeutic intervention (Nag et al., 2014).

Attention has been drawn towards strategies to inhibit MDM2 activity. Anti-MDM2 therapy might not only re-establish p53 activity in tumours with amplified MDM2 genes, but it might re-establish p53 activity in wild-type p53-expressing tumours with normal levels of MDM2 (Zhang et al., 2014). Nutlins, the p53-MDM2 binding inhibitors, activate the function of p53 causing arrest of cell cycle together with cancer cell apoptosis (Park et al., 2013).

Several studies focused on antiangiogenic agents, inhibitors for poly(adenosine diphosphate [ADP]-ribose), inhibitors to insulin growth factor receptor and targets for epidermal growth factor receptor/human epidermal growth factor receptor , together with antagonists to folate receptor to investigate their potential use as targeted

therapy in epithelial carcinomas of ovary (Coward et al., 2015).

Other studies addressed search for biomarkers helping in biologic stratification of ovarian carcinomas (Kobel et al., 2016). CUEDC2 has been declared as promising in predicting relapse in ovarian serous carcinomas (Wang et al., 2015). Understanding the molecular mechanisms of epithelial ovarian carcinomas may assist in detecting prognostic biomarkers and new targets for therapy such as miR-106b (Chen et al., 2015) and HtrA2 (Miyamoto et al., 2015). A year later, CA125 was mentioned as the best current biomarker for routine use in those tumours (Gyorgy et al., 2016).

AIM

The aim of this study was to examine the immunohistochemical expression profiles of MDM2 oncogene protein in the ovarian serous and mucinous epithelial tumours (benign, borderline and malignant).

Materials and Methods

A total of forty five paraffin blocks of ovarian tumour cases was retrospectively retrieved from the pathology department at Kasr El-Aini hospitals. Ovarian tumour blocks included fifteen cases of benign cystadenomas (5 serous & 10 mucinous), fifteen cases of borderline tumours (11 serous & 4 mucinous) and fifteen cases of cysadenocarcinomas (9 serous & 6 mucinous). The age of the cases ranged between 21 and 75 years with a mean age of 53.15 years.

Two sections 4µm thick each, were cut from each block: one section was stained with hematoxylin and eosin (H&E) for routine histopathologic study. The other section was immunostained according to (Kanthan et al., 2010) with mouse monoclonal anti-MDM2 antibody (clone SMP 14, Abcam (ab3110); 100µg at 1mg/ml) using dilution of 1:50 for 30 minutes overnight at temperature 4°C. Ultravision detection system (Lab Vision Corporation (Tp-015-HD), UK) was used. Paraffin sections were cut from the paraffin block of a known breast carcinoma, immunostained with MDM2 for positive control. Other sections of ovarian carcinoma cases; omitting the primary antibody and using diluent instead; were used as negative controls. One negative and one positive control were used in each run.

Immunostaining results were interpreted according to the percentage of positive tumour cells being evaluated in 10 fields at the magnification X 200; this denotes the extent of MDM2 immunostaining. It was performed at the Pathology Department, National Research Centre using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England,). Tumour cells showing distinct nuclear or cytoplasmic or both nuclear and cytoplasmic staining were considered positive according to (Hav et al., 2011). Immunostaining Extent was scored as follows: score 0 (negative): 0-5% positivity; score 1: 6-49% positivity; score 2: ≥50% positivity) according to (Lee et al., 2005). The immunostaing intensity of the tumor cells was classified into: weak, moderate, and strong according to (Kanthan et al., 2010)

Table 1. Relation between MDM2 Positivity and Tumour Category in the Studied Ovarian Tumours

MDM2 expression	Benign	Borderline	Malignant
Positive	-	3 (20%)	10 (66.7%) ^a
Negative	15 (100%)	12 (80%)	5 (33.3%)
Total	15 (100%)	15 (100%)	15 (100%)

Positive: Immunostaining present; Negative: No immunostaining; a: Positivity of MDM2 significantly higher in malignant than borderline tumours (P value of chi-square test =0.000)

and interpreted semiquantitatively into an intensity scoring system where: 0= no detectable immunostaining; 1= weak or faint cytoplasmic and/or nuclear immunostaining; 2= moderate cytoplasmic and/or nuclear immunostaining; 3= strong cytoplasmic and/or nuclear immunostaining. Final score was obtained by multiplying immunostaining extent by immunostaining intensity according to (Salim et al., 2008).

Statistical analysis

Statistical analysis of immunohistochemical results was performed using the chi-square test and Z test with a P value set as <0.05 to indicate significance.

Results

In this study, a total of forty five paraffin blocks of selected ovarian tumour cases was retrospectively retrieved from the pathology department at Kasr El-Aini hospitals. The studied ovarian tumour cases were classified as: fifteen cases of benign cystadenomas (5 serous & 10 mucinous), fifteen cases of borderline tumours (11 serous & 4 mucinous) and fifteen cases of cystadenocarcinomas (9 serous & 6 mucinous). Serous type of the studied ovarian tumours constituted 25 cases (55.6%), while mucinous type constituted 20 cases (44.4%). (Figure 1).

The MDM2 positivity was detected in thirteen (28.9%) out of the forty-five studied cases of ovarian tumours. All the studied benign tumours were negative for MDM2. Three (20%) of the studied borderline tumours expressed MDM2 (all cases were serous, score 1; two cases showed weak staining & one case showed moderate staining), and all borderline mucinous cases were negative). Ten (66.7%) of the studied malignant tumours expressed MDM2 (five cases at score 2 & five cases at score 1; five cases were of serous type & five cases were of mucinous type; three cases showing strong, two cases showing moderate & five cases showing weak intensity). The positivity of MDM2 was significantly higher in the malignant than the borderline tumours (P value of chi-square test =0.000) (Table 1), (Figure 2).

The correlation between MDM2 positivity and the different histologic types (serous & mucinous) among the borderline category was as follows:

MDM2 expression was negative in eight cases (72.7%) of borderline serous tumours and positive in three cases (27.3%) of them with a significant difference between positive and negative cases (P value of Z test =0.04). MDM2 expression was negative in all cases (100%) of borderline mucinous tumours (Table 2).

The correlation between MDM2 positivity and the different histologic types (serous & mucinous) among the

malignant category was as follows:

MDM2 expression was positive in five cases (83.3%) of malignant mucinous tumours and was negative in one case (16.7%) of them with a significant difference between positive and negative cases (P value of Z test =0.01).

MDM2 expression was positive in five cases (55.6%) of malignant serous tumours and was negative in four cases (44.4%) of them with an insignificant difference

Table 2. Relation between MDM2 Positivity and Borderline Category in the Studied Ovarian Tumours

MDM2 expression	Serous Borderline	Mucinous Borderline
Positive	3(27.3%)	-
Negative	8(72.7%) ^b	4(100%)
Total	11(100%)	4(100%)

Positive: immunostaining present either nuclear or cytoplasmic or both nuclear and cytoplasmic; Negative: no immunostaining neither nuclear nor cytoplasmic; ^bshowing significant difference from positive cases (P value of Z test =0.04).

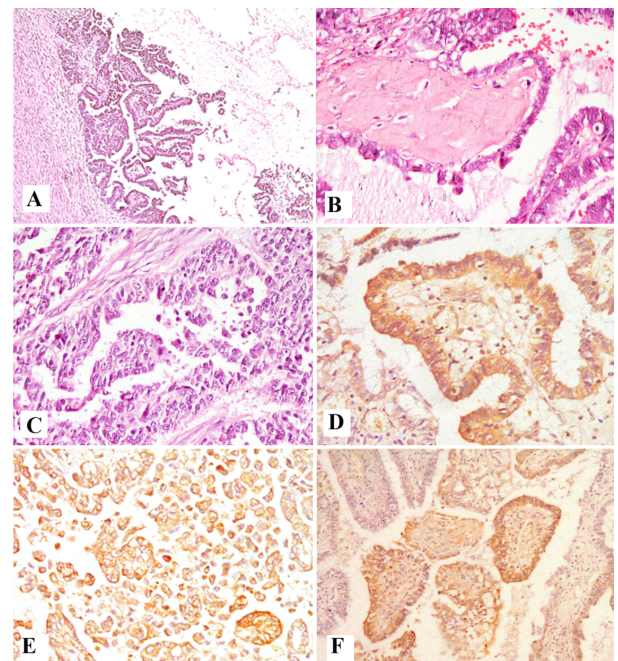


Figure 1. Histology and Immunohistochemistry Findings. (A) Borderline serous tumour showing surface papillae lined by stratified cuboidal to columnar epithelial cells (H&E, X100). (B) Borderline serous tumour showing papillae lined by stratified cuboidal to columnar cells with mild nuclear atypia (H&E, X 400). (C) Serous cystadenocarcinoma showing stromal invasion (H&E, X400). (D) Borderline serous tumour showing moderate cytoplasmic MDM2 immunostaining, (Immunoperoxidase, X 400). (E) Serous cystadenocarcinoma showing moderate cytoplasmic MDM2 immunostaining, (Immunoperoxidase, x 200). (F) Borderline serous tumour showing moderate cytoplasmic MDM2 immunostaining (Immunoperoxidase, X 200)

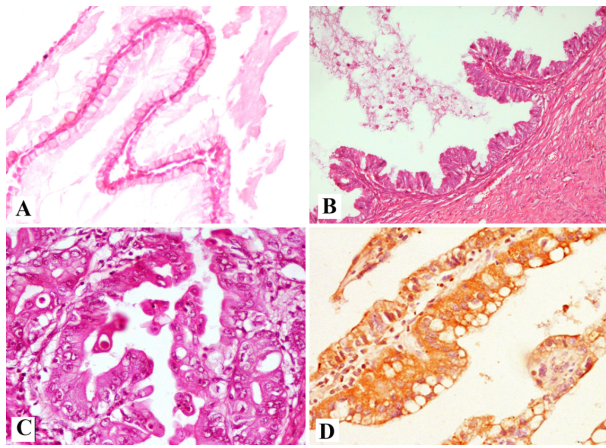


Figure 2. Histology and Immunohistochemistry Findings. (A) Benign mucinous cystadenoma showing nonstratified columnar epithelium with abundant, pale-staining intracellular mucin and small, basal nuclei (H&E, x 200). (B) Borderline mucinous tumor showing papillary infoldings lined by stratified epithelium with mild atypia (H&E, X 200). (C) Mucinous cystadenocarcinoma showing marked cytological atypia (H&E, X 400). (D) Mucinous cystadenocarcinoma showing strong cytoplasmic MDM2 immunostaining (Immunoperoxidase, x 400)

Table 3. Relation between the Extent of MDM2 Immunostaining and Tumour Categories

Tumour Category	Total number of cases	MDM2 Immunostaining Extent		
		0 (negative)	1+	2+
Benign	15	15	-	-
Borderline	15	12	3	-
Malignant	15	5	5	5

score 1+: 6-49% positivity; score 2+: $\geq 50\%$ positivity

Table 4. Relation between the Final Score of MDM2 Immunostaining and Tumour Categories

Tumour Category	Total number of cases	Final Score					
		0 (negative)	1	2	3	4	5
Benign	15	15	-	-	-	-	-
Borderline	15	12	2	1	-	-	-
Malignant	15	5	3	-	2	2	3

Final score: immunostaining extent $\times$ immunostaining intensity

between positive and negative cases (P value of Z test = 0.38)

A percentage of 62.5% of MDM2 positive serous tumours were of malignant category while 37.5% of them were borderline. All MDM2 positive mucinous tumours were malignant and there were no positive mucinous tumours of borderline category.

There was a highly significant statistical difference between the three tumour categories in relation to the MDM2 immunohistochemical extent scoring (P value of chi-square test = 0.000) (Table 3).

Discussion

In this study, we examined the immunohistochemical expression profiles of MDM2 oncogene protein in the ovarian serous and mucinous epithelial tumours (benign, borderline and malignant).

The MDM2 positivity was detected in thirteen (28.9%) out of the forty-five studied cases of ovarian tumours. These results were nearly similar to the results of (Dogan et al., 2005) where the MDM2 positive staining was detected in 32.9% (27/82) of the patients. Detection of MDM2 positivity in ovarian tumours may be used in therapy; where antagonists to MDM2 can accentuate the ability of cisplatin in causing apoptosis. It can also beat chemoresistance; as declared by (Mir et al., 2013).

In our study, we found that cytoplasmic MDM2 expression was seen in 20% of the cases; both nuclear and cytoplasmic MDM2 expression was present in 8.9% of the cases while no cases showed nuclear MDM2 expression only. We followed interpretation of MDM2 positivity adopted by (Hav et al., 2011), where tumour cells that showed distinct nuclear or cytoplasmic or both nuclear and cytoplasmic staining were considered positive. In their study, cytoplasmic expression was present in 36% of the cases, both nuclear and cytoplasmic MDM2 expression was seen in 11% of the cases whereas 23% of cases showed nuclear MDM2 expression. Also, in the study of (Lee et al., 2005) tumour cells that showed nuclear or cytoplasmic staining were considered positive. Meanwhile, (Turbin et al., 2006) detected nuclear staining in very few cases, and most positive cases showed only cytoplasmic staining; that's why at interpretation cytoplasmic staining only was considered. Cytoplasmic expression of MDM2 protein was demonstrated by (Hav et al., 2011) as true and needs to be considered in MDM2 immunohistochemistry work. In contrast, Tachibana et al. (2003) considered the cell as an immunohistochemically positive cell, if the nuclear staining was more intense than the cytoplasmic staining. Also, Palazzo et al., (2000) told that tumour cells that showed distinct nuclear staining were considered positive. This variation in MDM2 cellular localization representing a controversy in the interpretation of MDM2 immunoreactivity; can be explained by MDM2 shuttling between the cytoplasm and the nucleus. Inside the nucleus, MDM2 binds to p53 transporting it to a proteasome in the cytoplasm. This allows its degradation (Jenkins et al., 2012).

In the present study, all the studied benign tumours were negative for MDM2. This was consistent with the study of (Cho et al., 2006) where they found no MDM2 staining in any benign tumours. On the other hand, (Palazzo et al., 2000; Lee et al., 2005) showed that nine (56.2%) and twenty-nine (59%) benign ovarian cystadenomas respectively stained positively for MDM2. The differences in the results may be due to using different antibody clones, as MDM2 (clone SMP14) was used in our study which was the same clone used in the studies of (Cho et al., 2006); while MDM2 antibody (clone 1B10) was used in the study of (Palazzo et al., 2000).

In the current study, a percentage of 66.7% (10/15) of the carcinomas expressed MDM2, and 20 % (3/15) of borderline tumours expressed MDM2. So the positive rate of MDM2 was significantly higher in malignant tumours than in borderline tumors (P value of chi-square test = 0.000). This came in agreement with (Skomedal et al., 1997) who demonstrated that 13% ovarian carcinomas at stage 1 and 4% ovarian borderline tumours showed

MDM2 protein overexpression. Our results were also consistent with the studies of (Tuna et al (2003; Tuna et al., 2004) which reported that MDM2 expression was a marker for tumour advanced stage. On the other hand, Palazzo et al. (2000) reported that the MDM2 immunopositivity was significantly higher in borderline tumours than in malignant ones. In their study, 90% and 70% of ovarian borderline and carcinomas respectively expressed MDM2. This controversy of the results can be explained by the difference in interpretation of MDM2 positivity, as they only considered tumour cells that showed distinct nuclear staining as positive. While in the present study, tumour cells that showed distinct nuclear or cytoplasmic or both nuclear and cytoplasmic staining were considered positive.

In the present study, MDM2 expression was positive in 55.6%(5/9) of malignant serous tumors, which was nearly similar to the study of (Cho et al., 2006) where they found that 46.8% of serous carcinomas showed MDM2 immunopositivity. We also found that MDM2 expression was positive in 83.3% (5/6) of malignant mucinous tumors with a significant difference between positive and negative cases (P value of Z test =0.01). This finding prompts further investigation in this area, focusing on MDM2 as a potential target for therapy.

The MDM2 immunohistochemical extent scoring system used in the present study, was similar to that used in the study of (Lee et al., 2005) where immunostaining was scored as: (score 0: 0-5% positivity; score 1: 6-49% positivity; score 2: ≥50% positivity). Among our cases, all benign tumours were score 0; in borderline tumors 80 % (12 cases) were score 0 and 20% (3 cases) were score 1; while in malignant tumours about 33.3% (5 cases) were detected in each score level (5 cases were score 0, 5 cases were score 1 and 5 cases were score 2). While in (Lee et al., 2005) study, 41%(20 cases) benign tumours were score 0, 20% (10 benign cases) were score 1 and 39 % (19 benign cases) were score 2: whereas 35 % (11 borderline cases) were score 0, 16 % (5 borderline cases) were score 1 and 48 % (15 borderline cases) were score 2 and 71% (68 malignant cases) were score 0, 18% (17 malignant cases) were score 1 and 11%(11 malignant cases) were score 2.

Among the extent scoring systems applied in different studies for MDM2 immunohistochemical expression, some systems were similar to the scoring system used in the current study; in which immunostaining was scored on a three-tiered scale e.g.: Tachibana et al.(2003) scored staining for MDM2 as 0 in case<20% cells showed immunopositivity; 1 in case 20—50% cells were immunopositive; and 2 in case >50% tumour cells showed immunopositivity. Other scoring systems divided the extent of immunostaining into four categories e.g. (Baekelandt et al., 1999):-, none (no immunoreactive cells); +, less than 5% of the cells showing immunoreactivity; ++, 5% to 50% of the cells showing immunopositivity; and +++, more than 50% of the cells being immunopositive.

We also divided immunostaining intensity for MDM2 into three levels (weak, moderate and strong). This is in agreement with (Foulkes et al., 1995; Kanthan et al., 2010; Hav et al. ,2011); where the intensity of immunostaining of the tumor cells was qualitatively scored as: weak, moderate, and strong. In our work, we detected the strong

intensity only in carcinomas, while moderate and weak intensities were detected in both borderline tumors and carcinomas.

Absence of a standard system for determination and interpretation of MDM2 immunopositivity and scoring may be one of the causes of the discrepancies in the results of MDM2 various studies. Thus, we need to establish a standard definition for interpretation of MDM2 immunopositivity and a standard system for scoring to facilitate the application of MDM2 immunohistochemical technique in ovarian carcinomas; as targeting MDM2 could be a new approach in cancer therapy.

The present study agrees with (Gamal el Din et al., 2015); in that light microscopy sections and MDM2 immunohistochemistry need assisting adjuvant methods as DNA ploidy to distinguishing borderline ovarian tumours that are most likely to behave aggressively.

In view of the significant increase in the positivity of MDM2 through borderline up to malignant tumours; we suggest that MDM2 oncogene protein may be useful as a marker for risk stratification and identification of patients with cancer development and progression in ovarian epithelial serous and mucinous carcinomas.. We also suggest further working on MDM2 immunohistochemical study , in conjunction with adjuvant methods as DNA ploidy and FISH, to detect MDM2 gene amplification; in an attempt to differentiate between the three tumour categories; benign, borderline and malignant. We recommend increasing the sample size in the future studies, focusing on the mucinous type and to examine the immunohistochemical expression profiles of MDM2 in the other types of surface epithelial ovarian tumors (endometrioid, clear cell, transitional, squamous, mixed and undifferentiated type).

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