RESEARCH ARTICLE

Immunohistochemical expression of p53 in Type I and II epithelial ovarian cancer among Sudanese women: a cross-sectional study [version 1; peer review: 1 approved with reservations]

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Abstract

Background: Epithelial ovarian cancer (EOC) represents the leading cause of death from gynecologic malignancies worldwide. In Sudan, ovarian cancer represents the fourth most frequent tumors among females. *TP53* somatic mutations is a defining feature of ovarian high-grade serous carcinoma. However, *p53* sequencing is not feasible in most low- and middle-income countries, like Sudan, and its frequency varies greatly. The study aimed to determine the frequency of *p53* overexpression and its relationship with tumor types I and II and tumor grade among Sudanese women with EOC.

Methods: In this cross-sectional, hospital-based study a total of 114 paraffin-embedded tissue blocks previously diagnosed as epithelial ovarian cancer were collected from six governmental hospitals in Khartoum state, Sudan, in the period 2013-2016. Immunohistochemistry was performed on tissue microarray slides to measure the protein expression of *p53* in the EOC.

Results: Overexpression of *p53* was detected in 35.1% (n=40/114) of EOC samples, with a higher frequency in women with Type II 53.7% (n= 29/54) than type I 18.5% (n= 10/54) (P= 0.000). Also, a high frequency of *p53* overexpression was evident in 49.2% (n= 30/61) of high-grade carcinoma compared with 16.7% (n= 1/6) of non-graded borderline tumors, and in 19.1% (n= 9/47) of low-grade tumors (P= 0.003). A high-grade serous carcinoma harbor *p53* overexpression in 53.7% (n= 29/54) and none of low-grade serous carcinoma harbor *p53* overexpression. Our result showed a significant association between

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1. Daniel R Barnes , University of Cambridge, Cambridge, UK

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p53 overexpression and tumor types and grades ($P = 0.000$ and 0.003 , respectively)

Conclusion: p53 over-expression was detected in one-third of Sudanese women with EOC. It was more common in type II EOC and high-grade serous, but negative in low-grade serous tumors. Our result showed a significant association between p53 over-expression and tumor type and grade, and can help discriminate between high- and low-grade serous carcinomas.

Keywords

Epithelial ovarian cancer, cross-sectional study, Immunohistochemistry, p53overexpression, Sudan

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Introduction

Ovarian cancer is a fatal disease, the mortality rate ranks the highest of all gynecological malignancies¹. It is considered the third most common cancer in the female reproductive system (following uterine cervix and corpus) and the leading cause of death from gynecologic malignancies in the United States as estimated by the American Cancer Society for the year 2019^{1,2}. Ovarian cancer refers to a group of morphologically and genetically heterogeneous neoplasms^{3,4}. In Sudan, ovarian cancer represents the fourth most frequent tumor type in women⁵.

Based on clinicopathological and molecular studies, epithelial ovarian cancer (EOC) is classified as type I or type II. Type I tumors are genetically quite stable, typically present at a low stage, and reveal distinct, morphologic differences than type II tumors⁶. These include different histotypes: low-grade serous, endometrioid, clear-cell, and mucinous ovarian carcinoma. Type I tumors are characterized by distinct molecular genetics profiles, such as mutations in *KRAS*, *BRAF*, *PIK3CA*, *PTEN* and *ERBB2*, but not *TP53*⁶. Type II tumors are generally high-grade serous (about 90% of all EOCs). They are highly aggressive, develop rapidly, present in an advanced stage in most cases, genetically unstable and express a mutated *TP53*^{7–12}. *TP53* mutations have an important role in the prognosis and treatment of ovarian cancer¹³. Mutations in *TP53* are found in high grade and rarely in low grade serous ovarian cancers. *TP53* encodes the 53 kDa nuclear protein, their mutations leading to gain or loss of function of its protein product. *TP53* mutation leads either to overexpression of p53 protein or complete lack of expression, while wild-type p53 is associated with focal expression^{14,15}.

Immunohistochemical staining for p53 was considered as an essential biomarker for clinical trials targeting mutant p53 and used in the diagnostic workup of carcinomas of multiple sites, including ovarian cancers¹⁶. It is used as a substitute for *TP53* mutational analysis, these mutations were global in high-grade serous ovarian cancer (HGSOC) (over 96% were mutated), so were used to discriminate between high- and low-grade serous carcinomas^{17–20}. Access to *TP53* sequencing is not feasible in many low- and middle-income countries; pathologists there used p53 immunohistochemistry, which is quick, easy to perform, inexpensive and can approach 100% specificity for the presence of *TP53* mutation. Its high negative predictive value is clinically useful as it can exclude the possibility of a low-grade serous tumor^{18–20}. To our knowledge, there are no published reports about the frequency of p53 immunostaining in type I and II EOC in Sudan. The study aimed to determine the frequency of p53 overexpression and its relationship with tumor types I and II and tumor grades among Sudanese women with EOC.

Methods

Study background

A cross-sectional, hospital-based study was implemented. All 114 available formalin-fixed paraffin-embedded tissue blocks (convenience sampling) previously diagnosed as epithelial ovarian cancer were collected during the period 2013–2016,

in six governmental hospitals in Khartoum state, Sudan (The National Public Health Laboratory, Maternity Hospital, Military Omdurman Hospital, Alribat, Bahri, and Omdurman Teaching Hospital). Well-preserved tissue blocks with adequate tissue left for tissue microarray (TMA) procedure were included. Inadequate tissue blocks, and cases with missing tissue blocks were excluded. Slides from the original paraffin blocks were stained with hematoxylin and eosin (H&E), were reviewed according to 2014 WHO classification of ovarian tumors²¹, and were graded and typed according to the Kurman model⁸.

Construction of a microarray

Available paraffin-embedded blocks from tumors were used for the construction of a tissue microarray (TMA). Representative areas of the tumor were identified and TMA blocks were constructed using two cores from each case. Sections were obtained from each TMA and were placed on negatively charged slides for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed to measure the protein expression of p53 monoclonal antibodies in ovarian carcinoma cases, as follow: Sections were cut into widths of 3–4 µm and placed on clean, electrostatically charged glass slides. Sections were dried by placing on a hot plate at 60°C for 15 minutes. Sections were dewaxed in two changes of xylene for two minutes. Sections were then hydrated through an ethanol series (100%, 90%, 70%, 50%) and water two minutes for each. Slides were retrieved using the water bath heat-retrieval technique²² and then treated with 3% hydrogen peroxide for 10 minutes. After that, sections were washed in phosphate buffer saline (PBS) (pH 7.4) for five minutes and treated with a 10% casein solution for 10 minutes. Sections were treated with ready-to-use primary antibody of mouse monoclonal antibody to p53 protein (clone DO7 IgG2b; catalog no AM239-5M; BioGenex, CA) for 30 minutes at room temperature in a humidity chamber, then rinsed in PBS before being treated with Super Sensitive polymer –HRP IHC Detection System (catalog no QD420-YIKE; BioGenex, CA) by incubated with enhancer reagents 15 min at room temperature, followed by a polymer-HRP reagent conjugated to anti-mouse and anti-rabbit secondary antibody for 15 minutes at room temperature, and rinsed in PBS. The entire antibody-enzyme complex is then made visible by incubation with a chromogenic substrate 3,3 diaminobenzidine for 7 minutes then washed in PBS for five minutes. For the staining step, sections were counter-stained in Mayer's hematoxylin for one minute washed and blued in running tap water before they were dehydrated through ascending concentrations of ethanol (50%, 70%, 90%, 100%). Sections were finally cleared in xylene and mounted using DPX. A known p53-positive breast cancer tumor was used as positive control. As the negative control, tumor specimens were immunostained under the same conditions without the primary antibody. Both the quantity of nuclear positivity and the staining intensity were measured in the immune slides examined under a light microscope (Olympus BX41, Japan). The intensity of staining was reported as negative, weak, moderate, or strong (0, 1+, 2+, 3+) in comparison with the positive controls (internal

or external) and it indicated the average staining intensity of the tumor nuclei on the entire slide. An IHC score of p53 staining intensity was categorized as 0 for none, (no brownish color seen using x40 magnification), +1 for weak (brownish color seen using x20 and x40), +2 for moderate (brownish color seen using x10 magnification) and +3 for strong staining (brown color visible using x4 magnification).

The percentage of positive tumor cells was quantified by counting cells manually in at least 100 cells in 10 high power fields, averaged and categorized as $\geq 75\%$ of cells considered as high overexpression, $\leq 75\text{--}50\%$ considered as moderate expression and less than 50% considered as focal expression. Only 75–100% positive tumor cells with moderate and strong staining intensity considered as positive results.

Statistical analysis

The data were analyzed using the statistical package for social sciences (SPSS version 24) to describe the variables. Pearson's Chi-square test was used to determine a statistically significant association between p53 expression and clinicopathological variables.

Ethical consideration

The study was approved by the ethical committees of Alzaiem Alazhari University and the Ministry of Health, Sudan. Informed consent from patients was waived by the committees, since patients' identity was anonymized, and only laboratory numbers were used.

Results

Grading and typing of samples

According to the histopathological diagnosis, all 114 cases were EOC. They were classified as type I, type II and borderline, in 47.4% (n=54), 47.4% (n=54) and 5.3% (n=6) of cases, respectively. Grade of samples was classified as high in 53.5% of cases (n=61), low in 41.2% of cases (n=47) and non-graded tumors in 5.3% of cases, respectively (n=6). EOC histological subtypes were high-grade serous (HGS) in 54 cases (47.4%), low-grade serous (LGS) in six cases (5.3%), mucinous carcinoma (MC) in 22 cases (19.3%), endometrioid carcinoma (EC) in 16 cases (14%), clear cell carcinoma (CCC) in seven cases (6.1%), malignant Brenner tumor in three cases (2.6%) and borderline in six cases (5.3%). Details of samples for each patient, alongside all unprocessed images, are available as *Underlying data*²³.

Overexpression of p53

Positive p53 immunostaining was seen in 35.1% (40/114) of ovarian epithelial carcinoma. From the 40 positive cases, staining intensity was as follows: 25 cases exhibited strong staining (+3), and 15 cases were moderate staining (+2). While the remaining 74 cases were considered as negative results (complete absence of p53 expression seen in 45 cases and +1 staining in 29 cases) (Figure 1–Figure 4).

Overexpression of p53 was associated significantly with the histological subtype ($p = 0.004$) as seen in Table 1. p53 expression was significantly associated with tumor grade

Figure 1. High-grade serous carcinoma showing strong immunostaining (+3 staining) for p53. Magnification: main image, x40; inset, x10.

Figure 2. High-grade serous carcinoma showed moderate immunostaining (+2 staining) for p53. Magnification, x40).

Figure 3. High-grade serous carcinoma showed weak immunostaining (+1 staining) for p53. Magnification, x40.

Figure 4. High-grade serous carcinoma showing absence of immunostaining for p53. Magnification, 40X.

Table 1. Association between p53 expression and histological subtypes of the studied ovarian tumors.

Histological sub-type	Variable	p53 expression		Total
		positive	negative	
High grade serous	n	29	25	54
	%	53.7	46.3	100%
Low grade serous	n	0	6	6
	%	0	100	100%
Mucinous carcinoma	n	7	15	22
	%	31.8	68.2	100%
Endometrioid	n	2	14	16
	%	12.5	87.5	100%
Clear cell	n	1	6	7
	%	14.3	85.7	100%
Brenner tumor	n	0	3	3
	%	0	100	100%
Borderline	n	1	5	6
	%	16.7	83.3	100%
P value		0.004		

($p = 0.003$): it was positive in 49.2% (30/61) of high-grade tumors, 16.7% (1/6) of the non-graded borderline tumors, and 19.15% (9/47) of the low-grade tumors. The association between p53 immunohistochemical expression and epithelial ovarian cancer type showed a highly significant association ($p = 0.000$). p53 was positive in 53.7% (29/54) of Type II and 18.5% (10/54) of Type I tumors (Table 2).

Discussion

Immunohistochemical staining for p53 was used as a surrogate for TP53 mutational analysis to discriminate between high (over 96% was mutated) and low-grade serous carcinomas^{17–20}.

Table 2. Association between p53 expression and studied ovarian tumor type and grade.

Clinicopathological characteristic (N)	p53 status		P value
	Positive	Negative	
Cancer Type			
Type I (54)	10 (18.5%)	44 (81.5%)	0.000
Type II (54)	29 (53.7%)	25 (46.3%)	
Borderline (6)	1 (16.7%)	5 (83.3%)	
Cancer Grade			
High grade (61)	30 (49.2%)	31 (50.8%)	0.003
Low grade (47)	9 (19.1%)	38 (80.9%)	
Non-graded (6)	1 (16.7%)	5 (83.3%)	

In the present study, p53 positivity was observed in 35.1% (40/114) of EOC cases, and higher positivity was showed in HGS (53.7%; 29/54); a significant association was found between p53 expression and histopathological diagnosis and with tumor grade and tumor type. This result agreed with those of Tan *et al.*, who found that 64.18% (43/67) of HGS samples analyzed over-expressed p53¹³. Harris *et al.*, found that in 274 cases, 68% of tumors were characterized as p53 mutant ($n = 186$) and p53-mutant tumors were more likely to be HGS (72%) with significant association between p53 expression and histology and grade of tumor¹⁰. Markowska *et al.*, found that positive expression of p53 protein was observed in 27.8% of EOC²⁴. Yemelyanova *et al.* found out of the 57 tumors, 36 contained functional mutations and 23 cases (63%) with mutant *Tp53* were positive for p53 IHC and 70% of HGS were p53 mutant¹⁶. Kobel *et al.* showed p53 marker overexpressed in 69% (118/171) of HGS cases¹⁸. Kobel *et al.* also showed that abnormal *Tp53* expression was detected in 904 out of 912 (99.1%) of HGS²⁵. Our results also agreed with those of Matsuo *et al.*, who found that for 121 cases of high-grade serous p53 positivity was (71.4%)²⁶, and Zhang *et al.*, found that strong staining of p53 was observed in (70.8%) of serous carcinomas and showed that the tumor type was closely associated with the expression of p53²⁷. Mozes *et al.*, found p53 overexpression in 83.6% of HGS²⁸. Bell *et al.* reported that *Tp53* mutated in 96% of HGS²⁹. Moreover, Oaknin *et al.* reported that expression of p53 markers in the various histological types of ovarian carcinoma (HGS 94%, LGS 0%, CCC 12%, EC 15% and MC 61%)¹⁷ were nearly to our results (HGS 53.7%, LGS 0%, CCC 14.3%, EC 12.5% and MC 31.8%). Lim *et al.*, reported that p53 was not expressed in EC in 30 samples, but they agreed with our result in that they found HGS overexpressed p53 in 50% of cases examined³⁰. Alexander *et al.* reported that p53 was positive for HGS in 68% of cases (52/76) and differed from our result in that they found positivity in LGS (24%; 18/76)¹⁹. Mackenzie *et al.*, reported a higher percentage of p53 in mucinous carcinoma (68 %)³¹.

Furthermore, our result agreed also with Sallum *et al.*, who reported that p53 positivity was found in 68.2% of HGS and significant association was found between p53 expression and

tumor type¹⁵. Sundov *et al.*,³² and Tan *et al.*,¹³ also found that immunoexpression of p53 was significantly associated with tumor grade. And it disagreed with Brachova *et al.*, who showed that p53 protein expression insignificantly associated with tumor grade³³.

The present study showed that p53 marker was overexpressed in (53.7%) of type II, and (18.5%) of type I. This result agreed with Carter *et al.*, 2018, who reported that p53 was highly expressed in type II EOC (68.8%) than type I (33.3%)⁴. HGSOc is the most frequent type of ovarian cancer and has been associated with a poor clinical outcome³⁴. According to The Cancer Genome Atlas report, mutations in *TP53* are the most common events in EOC, especially in HGSOcs³⁵.

The results of some of these studies may be conflicting primarily because of the indiscriminate grouping of *TP53* mutations, which can result in either loss of function or gain of function³⁶. GOF mutations can convert p53 protein from a tumor suppressor to an oncogene, leading to expression of a mutant p53 protein at a high level, while LOF mutants leading to loss of p53 protein expression³⁷. *TP53* mutations are classified according to their function as oncomorphic, loss of function and unclassified. Around 21% of all ovarian cancer patients harbor oncomorphic *TP53* mutations, which had the highest p53 protein levels and contribute to chemoresistance and cancer progression, and the tumors with unclassified *TP53* mutations express the mutated p53 protein at a fairly high level³³. The differences found between the studies in the frequency of p53 expression may be due to the differences in scoring of p53 expression and interpretation of results: some studies scored this as overexpression (OE), complete absence (CA), cytoplasmic (CY) or normal/wild type (WT)^{18,20}. Some authors consider complete absence of p53 expression as a mutant also because not all *TP53* mutations alter the expression of the protein^{15,17,25}. Complete absence of p53 expression does not indicate *TP53* mutation, as a lack of immunoexpression may be found in normal cells¹⁶. So, we believe that true overexpression (more than 75% of the cells stained positive) was the most important type of mutation to consider due to their importance in clinical practice, as they are chemoresistant mutations³³ and could also be interpreted easily in the immune-slide without any confusion.

Study limitations

The limitations of our study were related to the relatively small sample size. Many cases were found in the lab records but lacking tissue blocks, and some blocks contain less amount of tissue for TMA.

Conclusion

Our study showed that the overexpression of p53 tumor marker is associated with EOC, histological subtype and tumor grade, and found that high-grade serous tumors had a higher percentage of p53 expression in more than 50% of cases, while low grade serous was negative in 100% of the cases. We recommended the use of p53 immunohistochemical staining in the pathologic workup of ovarian carcinomas. Careful attention to laboratory protocols and practical works, including adequate controls, and training in interpretation is needed to make this a reliable test informing diagnosis and subsequent management of ovarian carcinoma.

Data availability

Underlying data

Figshare: p53 data.csv, <https://doi.org/10.6084/m9.figshare.990130723>.

This project contains the following underlying data:

- p53 analysis in ovarian cancer (spreadsheet containing details of analysis for each patient sample).
- Uncropped, unprocessed images taken during this study.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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GENERAL COMMENTS/OVERVIEW

In this study, the authors present their findings from analysing p53 expression in a sample of 114 tissue microarrays obtained from Sudanese women diagnosed with ovarian cancer. They report statistically significant associations of differences in p53 expression and ovarian cancer histotype, grade and type.

The paper is generally well written, with all experiments and analyses well described.

MAJOR CONCERNS

Table 1: The chi-square test used to test the association between ovarian cancer subtype and negative/positive p53 expression is likely to be inappropriate, stemming from the small table cell counts (and even some zeroes). Here I feel that, although it is unlikely to make any difference to the reported association P-value and conclusions, a Fisher exact test is the correct statistical test.

Table 2: Similar to the comment on the statistical analysis for data presented in Table 1, a Fisher exact test would be more appropriate to analyse these data. The P-value presented for the association between cancer type and p53 status was "0.000". Could the authors present the P-value in scientific notation if it is $P < 0.001$?

Table 2: What was the magnitude of these associations? Could the authors fit logistic regression models to these data to estimate odds ratios of a p53 negative/positive expression by cancer type and cancer grade?

Discussion, first and second paragraphs: The authors discuss the findings of many other studies in lengthy detail, showing numbers and percentages. It is somewhat difficult to read. A more general overview with fewer details and providing references should suffice.

The authors rightly acknowledge the study sample size as a limitation in the discussion. Could they expand on what could be done in the future to increase sample sizes to strengthen evidence of associations and solidify the conclusions drawn.

The authors do not write a section on the strengths of this work. One point I feel that the authors should promote is the fact that this study investigated cancer genetics in an underrepresented population. The authors could make more of the fact that their study was conducted in women of African ancestry.

MINOR CONCERNS

Abstract, Results: The authors state a P-value of “P=0.000”, which could be more informative. Could the authors either state P

Introduction, first paragraph, first sentence: Could the authors clarify that the ovarian cancer mortality rate is highest worldwide (in agreement with what they say in the abstract).

Introduction, first paragraph, last sentence: Could the authors also state what the three cancers are that occur more frequently than ovarian cancer.

Introduction, second paragraph: The authors mention several genes involved in type I and type II tumours. I was interested to see that *BRCA1* and *BRCA2* were not mentioned. Would the authors be able to comment on the role of *BRCA1* and *BRCA2* in type I and type II ovarian cancers?

Methods, Immunohistochemistry, last paragraph (p4): The authors write “averaged and categorized as $\geq 75\%$ of cells considered as high overexpression, $\leq 75\text{--}50\%$ considered as moderate expression...”. The boundary at 75% is not clearly defined as they have greater than or equal to 75% in one category and less than or equal to 75% in the other category. Could they correct this to show which category includes 75%?

Methods, Statistical analysis: Was there a prior P-value specified for determining what constitutes a statistically significant association? Were there any multiple testing corrections made or considered?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Statistics; Genetic Epidemiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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