

Original Article

Implication of microRNA-regulated PTEN expression in the clinico-pathology & survival outcomes in advanced ovarian cancer

Ranita Pal^{1,4}, Sinjini Sarkar¹, Trisha Choudhury¹, Madhurima Ghosh¹, Manisha Vernekar², Partha Nath³ & Vilas D. Nasare¹

Departments of ¹Pathology and Cancer Screening, ²Gynaecological Oncology, & ³Medical Oncology, Chittaranjan National Cancer Institute & ⁴Department of Zoology, University of Calcutta, Kolkata, West Bengal, India

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Background & objectives: Phosphatase and TENs in homolog (PTEN), deleted on chromosome 10, plays a salient role in suppressing the proliferative phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signal in cancers. Growing evidence suggests that PTEN is downregulated by microRNAs (miRs) in aggressive cancers, which antagonise its tumour-suppressive activity. This study elucidates the effect of miR-214, miR-433, miR-100, and miR-152 on PTEN expression with important clinical parameters in individuals with Stage III-IV ovarian cancer (OC).

Methods: This prospective observational study enrolled 104 individuals with OC from January 2018 to December 2020. Demographic and clinical data were collected at presentation and follow up. Tissue samples were analysed using immunohistochemistry, Western blot, and quantitative real time PCR (qPCR)s. Statistical analyses included t-tests, chi-square, correlation coefficient, log-rank, Cox regression, and ROC analysis to assess clinical and survival outcomes.

Results: The included study participants with OC (mean age 49.29±9.68 yr) presented with advanced stages (96.6%) and had high-grade serous histological subtype (48.5%). Loss of PTEN expression was detected among 50.96 per cent, indicative of poor survival (HR>1; $P<0.05$). MiR-214 ($P<0.001$) and miR-433 ($P<0.001$) were negatively associated, while miR-100 ($P<0.001$) and miR-152 ($P<0.001$) were positively correlated with PTEN mRNA and protein, with miR-214, and miR-152 being independent risk factors to survival of OC patients (HR=>1). The sensitivity and specificity of PTEN and miRs range between 62.5-97 per cent, with diagnostic accuracy ($P<0.001$).

Interpretation & conclusions: The degree of miR-214, miR-433, miR-100, and miR-152 exhibited dysregulation in OC ($P<0.001$). The findings of this study suggest that miR-214 and miR-433 can downregulate PTEN whereas miR-100 and miR-152 may have a tumour suppressive role like PTEN. Thus, the signature miR network has the potential to become a diagnostic and prognostic biomarker.

Key words Biomarkers - miRNAs - ovarian cancer - PTEN - survival

Ovarian cancer (OC) is among the predominant lethal malignancies that impact the female reproductive

system, ranking as the 5th major cause of cancer-related death among women¹. Annually, around 2.2

lakh women receive a diagnosis of OC, resulting in about 1.40 lakh fatalities¹. In India, 26,834 new cases and 19,549 deaths are estimated to occur every year¹. The high mortality is attributed to the absence of early cancer-related symptoms and challenges in safe and minimally invasive methods for early detection. Therefore, around 40-50 per cent of cases of OC are identified in advanced stages (III and IV). In recent years, the breast cancer gene 1/2 (BRCA1/2) and homologous recombinant repair (HRR) deficiency investigations have been in clinical practice for the implementation of combined platinum and poly(ADP-ribose) polymerase inhibitors (PARPi) agents that affect first-line chemotherapy response². Even with decades of research advances, molecular intricacies and heterogeneity pose a hindrance to the development of effective diagnostic and prognostic biomarkers, along with effective therapeutic targets for OC.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) (PAM) pathway is an important tumorigenic signalling that also impacts chemo- and radiotherapy resistance in OC³. Phosphatase and TENs in homolog (PTEN) has been discovered as the pivotal negative regulator of the PI3K/AKT pathway and inhibits the parallel oncogenic pathways⁴. In malignant tumours like melanoma, glioblastoma, breast, thyroid, prostate, endometrial, ovarian, and colorectal cancers, PTEN is inactivated. Loss of PTEN happens *via* various mechanisms, such as multiple mutations, deletions, silencing through promoter hypermethylation, subcellular mislocalisation, changes in cellular stability, and protein half-life⁵.

Non-coding RNAs, such as microRNAs (miRNAs/miRs), represent a research hotspot in view of their effective importance in the post-transcriptional regulation of target gene expression that is linked with the cellular activity of cancer. They are associated with tumorigenesis, progression, diagnosis, and prognosis of cancer⁶⁻⁸. MiRs dysregulate expression of targeted genes at the post-transcriptional level by binding to the 3'UTR (3' Untranslated Region) of messenger RNAs (mRNA). Studies showed that several miRNAs influence the PI3K/AKT/mTOR (PAM) pathway,^{9,10} and hence, we hypothesised that the inactivation of PTEN will also be impacted by miRs. miR-152 and miR-214 were previously reported to regulate the expression of PTEN in prostate, nasopharyngeal, and gastric cancer¹¹⁻¹³. miR-100 and miR-433 modulate AKT/mTOR and exert tumour suppressive activity in

cervical cancer and glioblastoma^{14,15}. The differential expression of miRNAs (miR-214, miR-433, miR-100, and miR-152) is allied with differentiation, proliferation, apoptosis, resistance, and metastasis in different cancers, as well as in OC¹⁶⁻²¹. However, the diagnostic and prognostic utility of the loss of PTEN, or precisely the utility of miRNA-regulated PTEN expression, is yet to be established in clinical practice.

To replenish these gaps, this study focused on analysing the clinicopathological correlation of PTEN levels and microRNAs (miR-214, miR-433, miR-100, and miR-152) among OC patients.

Materials & Methods

This research was carried out in collaboration with the departments of Pathology and Cancer Screening, Gynaecological Oncology and Medical Oncology, Chittaranjan National Cancer Institute, Kolkata, West Bengal, India. The institutional ethical committee has approved this study, and informed consent was taken from all participants with the Declaration of Helsinki.

Study participant selection and sample collection: Newly diagnosed individuals with OC (n=104) who were treated from January, 2018 to December, 2020 were enrolled and clinicopathological characteristics were recorded. The inclusion criteria for selecting the participants were ≥ 20 yr who were diagnosed with epithelial OC and did not receive treatment; those with I-IV staging as per International Federation of Gynecology and Obstetrics (FIGO); predicted survival of patients must not be lower than one month; and participants willing to take part in the treatment process with follow up. The exclusion criteria was (i) history of other tumours or those who underwent complete oophorectomy and received any treatments like radiotherapy, chemotherapy previously, prenatal or nursing women; (ii) individuals with severe illness associated with hepatitis, active infections, or any other serious conditions of lungs, kidney, and heart and (iii) those with unrestrained substantial autoimmune ailment. A tissue sample (500 mg-1g) was obtained from the study participants during primary or interval debulking surgery, and normal ovary tissues were taken *via* resection as a control. Samples were collected in RNA Later (HiMedia, India) and 10 per cent neutral buffered formalin and preserved at -80°C for the study of mRNA, miRNAs, and protein expression (Supplementary Fig. 1).

Follow up and clinical response: The expression level of PTEN was considered as the primary predictor variable, whereas other characteristics (age, tumour stage, grade, size, treatment module, response, and miR expression) were taken as secondary predictor variables. The major outcome was overall survival (OS) and follow up was carried out telephonically until death or last follow up upto June 2023. As per our previous study,²² the clinical efficacy of OC patients was classified as Complete Responders (CRs), Partial-Responders (PRs), and Non-Responders (NRs).

Immunohistochemical expression of PTEN: An immunohistochemical (IHC) assay was performed for the expression of PTEN protein in the formalin-fixed and paraffin-embedded tissue specimens of the OC patients. This was done using the corresponding rabbit polyclonal antibody (AbCam, UK) with a dilution of 1:500 and secondary goat against rabbit IgG antibody (AbCam, UK) following the pre-treatment in citrate buffer in microwave (pH-6.0). The staining was visualised with 3,3'-diaminobenzidine tetrahydrochloride (DAB) followed by counterstaining with haematoxylin. The degree of immunostaining of PTEN cells was examined for the scoring which varied from 0-100 per cent, and a three-grade scoring system was used for evaluation of PTEN expression as (1) negative (0-10% stained cells); (2) + (11-50%); and (3) ++ (>50%)²³.

Western blot: Tissues were homogenised for 10 min in radio-immunoprecipitation assay (RIPA) lysis buffer. Cellular protein was obtained from cell pellets using the whole cell extract method in a protein lysis buffer that contains protease and phosphatase inhibitors. Protein concentration was checked and 50-100 µg proteins were separated on 10 per cent SDS-PAGE, and the gel was subsequently shifted onto a polyvinylidene fluoride (PVDF) membrane (Merck Life Science, USA). Then, the PDVF filter was immersed in the five per cent non-fatty milk in TBS-Tween (TBST) (pH 7.4) and incubated for 1-3 h at room temperature with antibodies against PTEN. Specific proteins were detected with primary antibody of Rabbit anti-PTEN (AbCam, UK), with 1:1000 dilution, incubated in a shaker for 1-3 h at room temperature or overnight at 4°C, and then washed with TBST thrice. The secondary goat anti-rabbit antibody was used at a 1:5000 dilution (AbCam, UK), followed by shaking at room temperature for 1-3 h. It was then washed with TBST thrice. Finally, visualisation of the protein band

was done through chemiluminescence (ECL) reagent (sc-2048; SANTA CRUZ Biotechnology, USA) and imaged with the ChemiDoc gel documentation system (Biorad, USA).

mRNA and miRNA expression detection: Tissues were homogenised for 10 min, and TRIzol (Invitrogen, Thermo Fisher Scientific, Inc., USA) was added for the extraction of total RNA. Nanodrop (Genetex, Korea) was used to determine the purity and concentration of the isolated RNA in its entirety. Using EasyScript™ (applied biological materials, Canada), reverse transcription of cDNA was done using the isolated RNA based on the manufacturer's instructions. Applied Biosystems 7500 Real-Time PCR equipment (Applied Biosystems; Thermo Fisher Scientific, Inc., USA) was used to assess the degree of mRNA (PTEN) and miRNAs (miRNA-214, miRNA-433, miRNA-100, and miRNA-152) using Low ROX-Evagreen 2xqPCR (Applied Biological Materials, Canada) from the synthesised cDNA. The sample comprised 10 µl of the SYBR green mix, 0.6 µl of ROX, ≤500 ng/reaction cDNA sample, and 0.6 µl of primer (forward and reverse) (Supplementary Table). Then, nuclease-free water was added to bring the volume to 20 µl. The reaction was conducted at 95°C for 10 min, then underwent 40 cycles for 10 sec at 95°C, 30 sec at 60°C, and 1 min at 72°C. All tests were performed in triplicate, normalising PTEN mRNA with 18S, and miRNAs with GAPDH, and the results were analysed by calculating the relative fold change ($2^{-\Delta\Delta Ct}$).

Statistical analysis: SPSS software (Version 26.0; IBM Corp., TX, USA) was used for the statistical analysis. Paired t-test analysis was done to check the differential expression of selected biomarkers in normal and OC tissue. Chi-square (Crosstabs) test was used to find the relationship between PTEN expression and clinicopathological parameters, and Pearson correlation coefficient was used to predict the association of miRs and PTEN. The survival was estimated using the Kaplan-Meier survival curve after monitoring the patients for three years, and the log-rank test was used for the comparison. It was also used to analyse survival associated with PTEN and miRNA expression. Cox regression was done to check the hazard ratio of death associated with various parameters. Receiver Operating Characteristic (ROC) analysis was performed to check the diagnostic value of PTEN and miRNAs. The criteria for statistical significance were $P < 0.05$.

Table I. Expression distribution of PTEN in association with various clinical parameters

Characteristics (n=104)	Percentage (%)	PTEN level (%)			P value
		1	2	3	
Age (yr)					
20-40	21.1	53.5	34.9	11.6	0.506
41-60	67.8	51.1	40.4	8.5	
61-80	11	65.2	21.7	13	
FIGO stage					
I & II	4.3	11.1	55.6	33.3	0.002
III	74.5	49.7	40	10.3	
IV	21.1	72.7	25	2.3	
Histology subtype					
Serous	70.5	33.8	27.5	9.2	0.17
Mucinous	15	19.3	12.8	0	
Clear cell	12.6	13.8	12.8	5	
Endometrioid	1.9	1.4	0.5	0	
Grade					
HGSC	48.5	51	39	10	0.041
LGSC	20.9	44.2	34.9	20.9	
Well differentiated	8.3	47.1	52.9	0	
Moderately differentiated	9.7	75	20	5	
Poorly differentiated	12.6	61.5	38.5	0	
Surgery					
Primary Debulking	49.6	85.3	13.3	1.3	<0.001
Interval debulking	50.4	32.8	52.3	14.8	
Clinical response					
Complete responders	34.6	33.3	45.8	20.8	<0.001
Partial responders	37.5	45.6	48.1	6.3	
Non responders	27.9	87.7	12.3	0	
Status at last follow up					
Alive	60.1	40.8	49.6	9.6	<0.001
Deceased	39.9	71.1	19.3	9.6	

PTEN level, 1-negative; 2-+; 3-++. P<0.05 is considered to be significant. HGSC, high grade serous carcinoma; LGSG, low grade serous carcinoma; FIGO, International Federation of Gynaecology and Obstetrics

Results

Clinicopathological features study: A total of 104 newly diagnosed OC patients were recruited in this study (Table I). The mean age was 49.29±9.68 years, ranging between 20-80 yr, and the majority were within the age group of 41-60 (67.8%). The larger portion of the participants were presented with advanced stages (74.5% and 21.1% FIGO stage III and stage IV, respectively), and a preponderance of them had serous carcinoma (70.5%) with high grade (48.5%) followed by mucinous (15.0%), clear cell (12.6%), and endometrioid (1.9%) EOC at the time of diagnosis. 49.6 per cent had primary debulking followed by adjuvant chemotherapy (ACT) and 50.4 per cent of participants underwent interval debulking surgery following neo-adjuvant chemotherapy (NACT). Response Evaluation Criteria in Solid Tumors (RESIST) and Gynecological Cancer Intergroup (GCIG) criteria-based evaluation categorised 34.6, 37.5, and 27.9 per cent of participants as CR, PR, and NRs, respectively. In addition, within the follow up period of 36 months, 39.9 per cent of the participants succumbed to the disease.

Association of PTEN expression with tumour progression and clinicopathologic parameters: The PTEN protein was found to be positive for all normal control tissues after IHC analysis in comparison to the malignant tissues (Fig. 1), in which only 15 per cent of cells showed nuclear expression. Whereas, in the case of OC, 11.15 per cent of cells had nuclear staining. Further stratification for OC based on staining intensity depicted 91.56 per cent, 87.66 per cent, and 86.25 per cent of cells had cytoplasmic staining with notably lower nuclear stain (Supplementary Fig. 2). Similarly, significant dysregulation in PTEN mRNA expression was found in the cancerous versus normal controls ($P=<0.001$) (Fig. 2A). Deficient PTEN expression was observed in 53 of 104 tumours (50.96%). The Western blots showed dysregulated expression of PTEN in cancer tissue with differential treatment (ACT/NACT) and normal tissue (Fig. 2B and Supplementary Fig. 3).

The chi-square test using crosstab analysis showed a weaker expression among individuals with stage IV (72.7%). Furthermore, in high-grade serous ovarian carcinoma (HGSOC) there were 10 per cent and 20.9 per cent to low-grade serous ovarian carcinoma (LGSOC) samples had high PTEN expression, and moderate expression (52.9%) of PTEN was detected in well-differentiated cancers, whereas minimal or

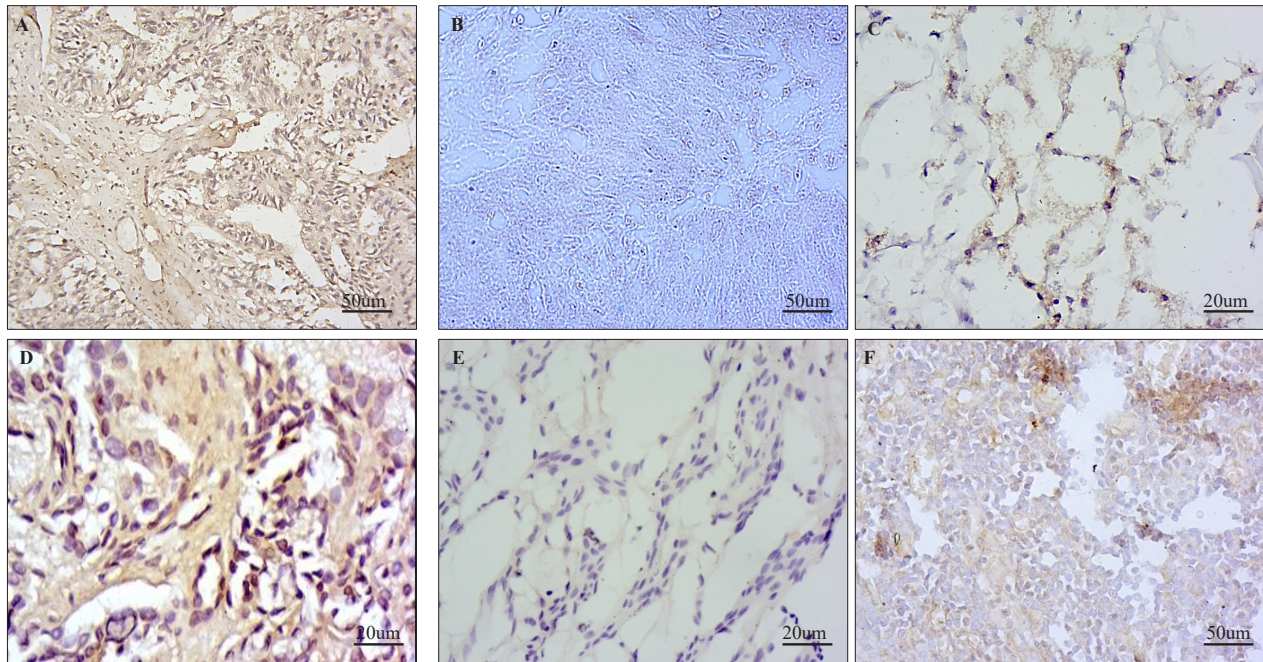


Fig. 1. Representative IHC staining of ovarian samples. (A) PTEN was positively expressed in normal tissue; (B) Negative expression for PTEN; (C-F) low-moderate PTEN expression was observed in some OC tissue samples.

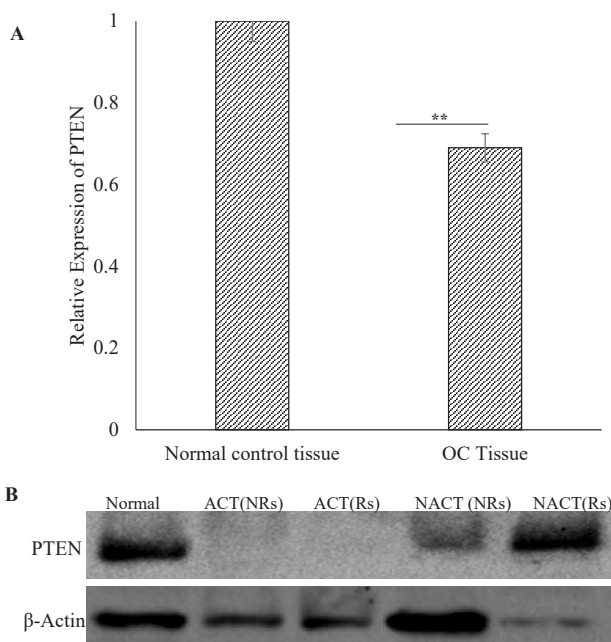


Fig. 2. Quantitative Realtime PCR and Western Blot analysis of PTEN messenger RNA (mRNA) and protein in individuals with ovarian cancer (OC). (A) The level of PTEN mRNA is significantly lower in cancer tissue than in normal tissue. (B) The level of PTEN protein (Normal: Lane 1; ACT: Lane 2, 3; NACT: Lane 4, 5) is dysregulated in the tissue of OC patients with different treatment responses compared with normal tissue. The $P^{**} < 0.001$ was considered significant. ACT, adjuvant chemotherapy; NACT, neoadjuvant chemotherapy; NRs, non-responders; Rs, responders.

negative expression (75% and 61.5%) was observed in moderate to poorly differentiated cancers, which suggested that PTEN expression may be regulated by the tumour microenvironment. Furthermore, the expression of PTEN protein did not have any significant association with age and tumour histology ($P \geq 0.05$) but was significantly correlated with clinical parameters such as stage ($P = 0.002$), grade ($P = 0.041$), mode of surgery ($P \leq 0.001$) of the tumour, and treatment outcome ($P \leq 0.001$) (Table I).

Correlation of PTEN and miRNAs dysregulation in OC: Paired sample t-test revealed that the levels of miR-214 and miR-433 were significantly elevated but miR-100 and miR-152 were suppressed in tumour tissues in comparison to normal tissues ($P \leq 0.001$) (Fig. 3). Nevertheless, the expression of miR-214, miR-433, and miR-152 suggestively varied between ACT and NACT cases ($P \leq 0.05$), though it was not relevant statistically for miR-100 ($P \geq 0.05$). Furthermore, the correlation coefficient test showed a strong positive association between PTEN mRNA and protein in OC ($r = 0.823$, $P \leq 0.05$) (Fig. 4A). Additionally, both PTEN mRNA and protein expressions are negatively correlated with miR-214 ($r = -0.453$, $P < 0.001$; $r = -0.542$, $P < 0.001$) and miR-433 ($r = -0.413$, $P \leq 0.001$; $r = -0.459$, $P \leq 0.001$) level; however, with the miR-100 ($r = 0.314$, $P \leq 0.005$; $r = 0.431$, $P \leq 0.001$) and miR-152 ($r = 0.344$, $P \leq 0.001$;

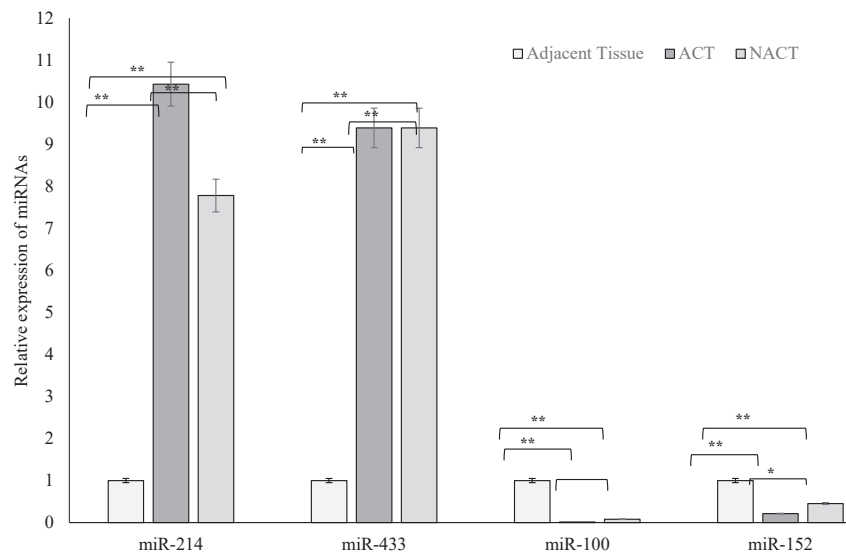


Fig. 3. Quantitative real time PCR analysis of miR-214, miR-433, miR-100, and miR-152 in individuals with OC. The level of miR-214 & miR-433 is significantly dysregulated in the tissue of OC individuals compared with normal tissue and with different treatments (ACT/NACT). The $P^* < 0.05$; $** < 0.001$ was considered significant.

$r = 0.354$, $P \leq 0.001$) exhibit positive association with PTEN mRNA and protein (Fig. 4B-C).

Overall survival and its association with PTEN and miRNAs dysregulation: The survival probabilities over time were estimated using the Kaplan-Meier estimator, and the log-rank test was employed to compare survival distributions among various patient groups. This test indicated a significant decrease in survival associated with age (15.71 months), tumour stage (15.6 months), grade (10.5 months), treatment response (18.54 months), PTEN expression (20.71 months), miR-214 (18.86 months), miR-433 (18.92 months), miR-100 (18.47 months), and miR-152 (19.60 months) expression ($P \leq 0.05$). Median survival times and corresponding 95 per cent confidence intervals (CIs) are reported in table II. Additionally, Cox proportional hazards regression was used to identify potential prognostic factors linked to OS. The Cox model was used for estimating hazard ratios (HRs) and 95% confidence interval (CI)s, adjusting for relevant covariates such as age, treatment type, pathological parameters, and biomarker expression levels. It showed age, tumour stage, grade, treatment, clinical response, PTEN level, and expression of only miR-214, and miR-152 are risk factors ($HR > 1$) for predicting survival among OC patients; however, it was not found to be statistical significance with age ($P = 0.001$) and treatment outcome ($P = 0.02$) (Table II). Furthermore, a concurrent comparative analysis of PTEN and

miRNAs presented that cumulative dysregulation in their expression levels had a considerable impact on the OS of the participants (Table III). Low PTEN levels with high expression of miR-214 (18) and miR-433 (12) were found to have poor survival ($P < 0.05$), while lower levels of PTEN alliance with declined miR-100 (16) and miR-152 (16), exhibiting worse survival ($P < 0.05$) (Table III). This suggests that the OS improved with the increased PTEN level.

The ROC analysis revealed a sensitivity of PTEN 91.11 per cent, whereas the specificity was 62.5 per cent. In addition, miRNAs showed sensitivity ranging between 91-97 per cent and specificity extending from 77-95 per cent. The area under the curve (AUC) of PTEN protein, miR-214, miR-433, miR-100, and miR-152 was 0.774, 0.983, 0.989, 0.912, and 0.995, respectively, with significant prognostic values ($P \leq 0.001$) to find efficacy between response groups (Table IV).

Discussion

PTEN, a frequently mutated tumour suppressor gene, is involved in numerous cellular processes, including proliferation, invasion, differentiation, cellular metabolism, genome stability, and apoptosis; thus, its alteration has a pivotal function in the development, recurrence, and metastasis of OC^{5,24}. The expression of PTEN can be regulated at several levels, such as genomic, transcriptional, post-transcriptional, and translational. Notably, several miRNAs have

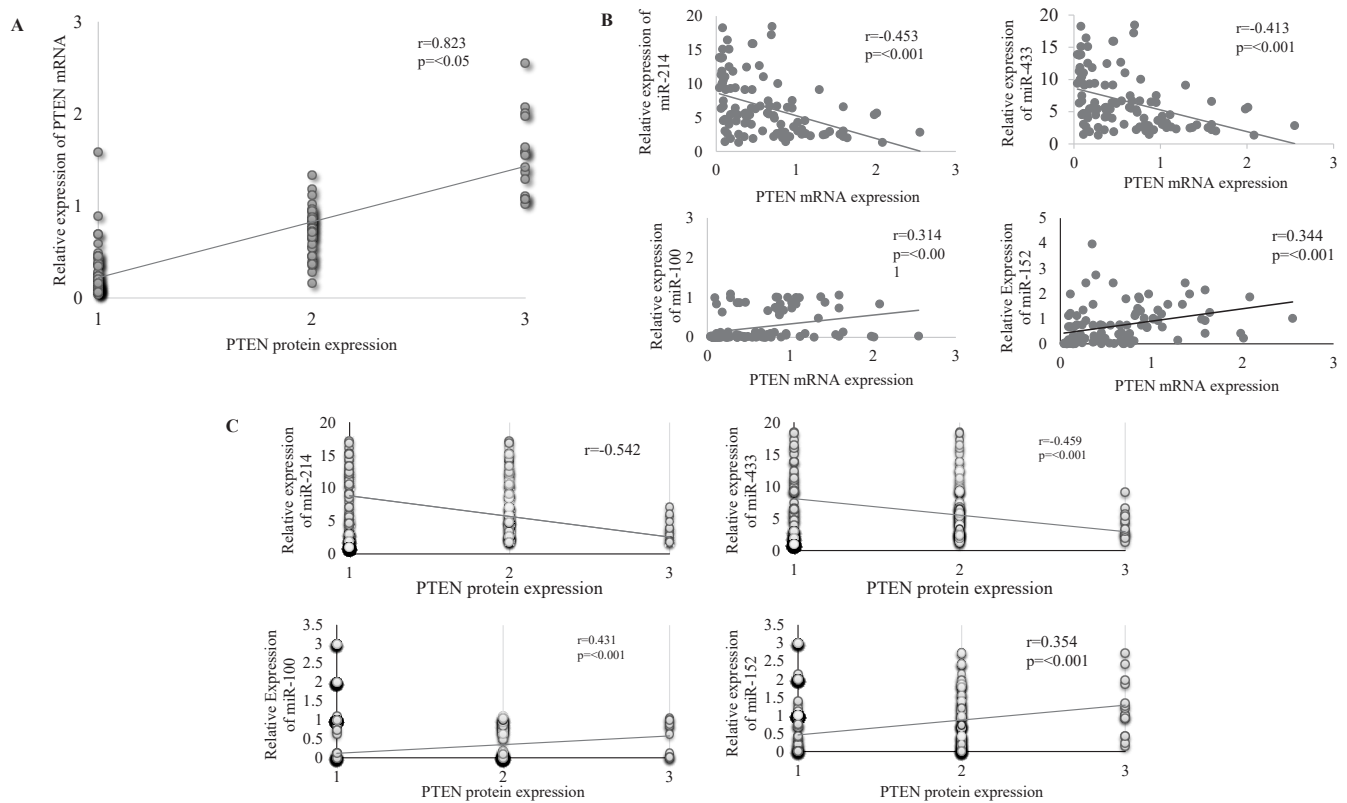


Fig. 4. Scatter plots for correlation analysis between PTEN protein, mRNA, and microRNA levels in ovarian carcinoma tissue samples. (A) between PTEN protein and mRNA; (B) between PTEN mRNA and miRNAs; (C) between PTEN protein and miRNAs. PTEN protein expression was divided based on immunohistochemistry score (1-negative; 2-+; 3-++).

been documented that dysregulate PTEN post-transcriptionally, which is linked with different critical aspects of cancers²⁵⁻²⁷. In recent years, both PTEN and miRs have shown increasing significance to clinical outcomes in different cancers; however, their clinical importance is not established yet to advance the early detection, prognosis, and treatment strategy.

Thus, the present study evaluated the levels of PTEN mRNA and protein in OC tissue to further analyse the molecular correlation with selected miRs (miR-214, miR-433, miR-100, and miR-152) and reported disease outcomes. Our study found that 49.29 ± 9.68 yr was the mean age of recruited patients presented with HGSOV (48.5%) of FIGO stages III and IV (74.5% and 21.1%). Similar data for age, higher stage, and serous grades were reported in a few studies²⁸. However, the higher mean age was recorded in diverse studies from around the world²⁹⁻³¹, which varied with our study. Our data showed a decreased level of PTEN in OC tissue compared to control tissue, which was consistent with various previously reported studies on OC^{26,32}. Correspondingly, this study showed

a significant association of PTEN expression in OC tissue with stage, grade, treatment mode, and outcome ($P < 0.05$) but not with age and size of the tumour ($P > 0.05$), which was comparable with the previous finding³³. This reiterates the previous findings that the downregulated expression of PTEN was related to the malignant transformation of ovarian tumours.

Furthermore, this study aimed to assess the influence of miRs on PTEN expression on the survival status of individuals with OC patients. Prior studies confirmed that the expression pattern of miR-214, miR-433, miR-100, and miR-152 is often altered in various cancer types, and few observations have been correlated with poor survival and treatment outcomes^{17-18,20,21,34-36}. The present study observed considerably increased relative expression of miR-214 and miR-433, but the miR-100 and miR-152 patterns decreased significantly in OC tissues in comparison to normal tissues. However, several studies found the dual function of miR-214 in quite a few cancers^{19,37-39} and most studies contrasted with our findings, which showed the down regulation of miR-433 in cancer^{20,35,40} except one¹⁶. In addition,

Table II. Univariate and multivariate analysis of clinicopathologic features for overall survival in individuals with OC

Clinicopathologic features (n=104)	Kaplan-Meier survival (univariate)			Cox regression survival (multivariate)		
	Survival (months)	95% CI	<i>P</i> value	Hazard Ratio	95% CI	<i>P</i> value
Age (yr)						
20-40	27.45	18.23-36.67	0.006	1.11	1.05-1.18	0.001
41-60	24.4	20.61-28.18				
61-80	15.71	11.16-20.26				
FIGO stage						
I&II	24.21	17.81-30.60	0.035	1.60	0.55-1.38	0.47
III	20.88	19.32-26.43				
IV	15.6	8.54-22.65				
Histology subtype						
Serous	23.44	18.44-28.45	0.17	1.10	0.80-1.52	0.54
Mucinous	16	8.77-23.22				
Clear Cell	22	12.51-31.48				
Endometrioid	25	23.04-26.96				
Grade						
HGSC	24.68	20.62-28.73	0.042	1.03	0.88-1.21	0.69
LGSC	21.57	13.66-29.47				
Well differentiated	10.5	1.68-19.32				
Moderately differentiated	23.2	15.26-31.13				
Poorly differentiated	21.4	12.79-30.00				
Surgery						
Primary Debulking	22.61	17.49-27.73	0.54	1.04	0.57-1.87	0.90
Interval debulking	24.42	20.72-28.12				
Clinical response (CR)						
Complete responders	31.42	25.25-37.59	0.006	1.47	1.06-2.02	0.02
Partial responders	26.57	20.95-32.18				
Non responders	18.54	14.85-22.22				
PTEN level						
1	20.71	17.33-24.08	0.039	1.87	0.53-1.41	0.58
2	29.25	22.38-36.11				
3	24.75	14.95-34.54				
miR-214						
Low	33.00	28.12-37.87	0.000	1.74	0.71-4.24	0.22
High	18.87	15.42-22.30				
miR-433						
Low	30.86	26.15-35.56	0.013	0.82	0.38-1.74	0.60
High	18.92	15.26-22.57				
miR-100						
Low	18.47	15.18-21.75	0.000	0.48	0.17-1.30	0.15
High	33.89	29.37-38.41				
miR-152						
Low	19.61	16.22-22.98	0.002	1.40	0.49-3.95	0.53
High	31.22	25.59-36.84				

PTEN level, 1-negative; 2-+; 3- ++. *P*<0.05 is considered significant. HR, hazard ratio; CI, confidence interval

Table III. Comparative expression analysis of PTEN with MicroRNAs in association to survival among advanced ovarian carcinoma individuals

Name of miRs	miRNAs expression	PTEN expression (%)			P value	Kaplan-Meier survival (univariate)						P value
		1	2	3		Survival time (months)			95% CI			
						1	2	3	1	2	3	
miR-214	High (n=51)	70.4	25.9	3.7	<0.001	18.00	21.66	18.53	14.84-22.22	8.57-34.76	6.24-29.76	0.005
	Low (n=53)	34.5	48.8	16.7		31.20	37.33	30.57	22.51-38.62	30.80-43.86	19.79-42.60	
miR-433	High (n=52)	67.1	29.3	3.7	<0.001	12.00	25.00	17.90	14.06-21.74	13.74-36.25	12.00-12.00	0.042
	Low (n=52)	37.3	45.8	16.9		30.00	38.50	28.54	21.73- 35.35	33.60-43.40	20.39-39.60	
miR-100	High (n=48)	32.	53.8	14.1	<0.001	36.00	36.00	31.00	24.09-37.91	27.48-44.51	27.68-44.31	0.004
	Low (n=56)	71.4	22	6.6		16.00	20.40	18.42	14.82-22.02	7.19-33.60	8.16-23.84	
miR-152	High (n=49)	34.2	50.6	15.2	<0.001	36.00	38.00	23.75	14.84-32.65	31.44-44.55	27.68-44.31	0.048
	Low (n=55)	70	24.4	5.6		16.00	21.00	19.66	15.93-23.38	8.57-33.42	8.16 -23.84	

PTEN level,1-negative; 2-+; 3- ++. *P* < 0.05 is considered to be significant

Table IV. Receiver operating characteristic (ROC) curves of PTEN and miRNAs (miR-214, miR-433, miR-100, & miR-152) for individuals with OC among different response group

PTEN and miRNAs	Sensitivity (%)	Specificity (%)	Youden index (J)	Area under the curve			Cut-off
				Area	95% CI	P value	
Responders (CRs+PRs) vs NRs							
PTEN	91.11	62.50	0.54	0.774	0.702- 0.846	<0.001	1.50
miR-214	93.30	88.46	0.87	0.983	0.968-0.997	<0.001	2.64
miR-433	97.78	95.00	0.93	0.989	0.975-1.000	<0.001	8.05
miR-100	91.11	77.50	0.67	0.912	0.870-0.955	<0.001	0.02
miR-152	97.78	95.83	0.94	0.995	0.989-1.000	<0.001	0.12

CRs, complete responders; PRs, partial responders; NRs, non-responders. *P* < 0.05 is considered significant

miR-214 and miR-433 were over-expressed in 70.4 per cent and 67.1 per cent of cancerous tissue that showed reduced or loss in the expression of PTEN, which indicated the negative correlation of miR-214 and miR-433 with PTEN. Besides this, 71.4 per cent and 70 per cent of tissues showed decreased miR-100 and miR-152 levels that were positively correlated with reduced or lost expression of PTEN and suggestive of tumour suppressive function like the target protein. These findings confirm that selected miRs are involved in regulating the expression of PTEN at OC. Notably, to date, several studies have disclosed a strong correlation of miR-214 with PTEN in various carcinomas, as well as in OC,^{12,13,26,37,39} but the association of PTEN with miR-433, miR-100, and miR-152 has not been well established in OC patients.

Additionally, the survival analysis of the present study indicated a vital prognostic influence of PTEN and miRNAs. In agreement with some studies^{33,41} our

results revealed that the age, tumour stage, grade, and depleted level of PTEN were significantly associated with poor OS and were an individual prognostic factor for OC. Moreover, this study found that higher expression of miR-100 and miR-152, and lower levels of miR-214 and miR-433 were also aligned with better prognosis in OC patients. Previous studies, along with other diverse observations on different miRNAs have shown the impact of miRNAs on better treatment outcomes and survival in OC^{22,42-44}. Thus, miRNA-214 and miR-152 might promote growth and invasion and could be considered as an effective marker to indicate the aggressiveness and poor prognosis of OC, as well as a potential therapeutic target.

The main limitations of this study were the small sample size from a single cancer centre and the detection of the PTEN and miRs from cancer tissues after an invasive procedure. The presence of confounding factors may have impacted our results,

showing a nonsignificant multivariate analysis. However, we plan to expand our research further and establish the same relationship through serum analysis to ease the diagnostic procedure, given that the ROC results showed AUC of 0.774, 0.983, 0.989, 0.912, and 0.995 for PTEN and miRNAs ($P < 0.001$) with acceptable sensitivity and specificity. In addition, PTEN mutational status was not analysed, and emphasis was not given to Stage I and Stage II OC pathology, which remains to be studied. To explore the mechanism of miR-214/miR-152/PTEN axis, more molecular assessments must be made. With the support of tissue microarray, a spectrum of miRs may be detected to be responsible for PTEN downregulation, which would have immense significance in elucidating the miRNA-regulated PTEN loss in OC. The lack of miRNA-mRNA correlation with treatment response is also a limitation of the present research.

However, this work represents one of the first reports of the clinical significance of collective miR-214/miR-152/PTEN expression patterns to prognosticate and predict survival outcomes in OC patients.

In conclusion, the study suggests that the aberrant expression of PTEN and miRNAs in malignant tissues may be a useful predictor of OC aggressiveness and survival. Furthermore, the correlation of PTEN with miR-214, miR-433, miR-100, and miR-152 had prognostic relevance, which proposes targeting the PTEN loss pathway regulated by miRs could be a promising therapeutic strategy for the treatment of OC.

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