ejpmr, 2021,8(11), 38-45

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EXPRESSION OF P16 PROTEIN IN SURFACE EPITHELIAL OVARIAN CARCINOMA & ITS ASSOCIATION WITH HISTOPATHOLOGICAL GRADING

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Article Received on 01/09/2021 **Article Revised on 22/09/2021** **Article Accepted on 12/10/2021**

ABSTRACT

Background: Malignant ovarian tumors are the seventh most common female cancer and one of the leading causes of morbidity & mortality. In Bangladesh, ovarian cancer ranks as the 4th most prevalent cancer. Surface epithelial ovarian carcinomas are the most common malignancy of ovary which constitute more than 90%. Prognosis and treatment protocol for ovarian carcinomas are depend on histopathological grade. p16 is a tumor suppressor gene which is a negative cell cycle regulator. Several studies have linked the role of p16 protein in ovarian tumorigenesis. In ovarian carcinoma p16 is either deleted, downregulated, or overexpressed. p16 is overexpressed in high grade epithelial ovarian carcinoma in comparison to low grade carcinoma. Expression of p16 is directly proportional to grading of the tumor and which could be a potential target for therapy. Keeping this on mind we aimed to investigate the immunohistochemical expression of p16 in ovarian carcinoma and to assess its association with tumor grade. **Materials and Methods:** This cross-sectional study was conducted in the Department of Pathology, Chittagong Medical College, Chattogram from March 2019 to February 2021. Forty-five Haematoxylin and Eosin stained slides of ovarian carcinoma cases were evaluated to find out their histopathological type, grade. Immunostaining was done by using primary antibody against p16 (Rabbit monoclonal, dilution 1:100). Patient's demographic data were collected and recorded in a predesigned data sheet. Statistical analysis was carried out as required. Ethical practice was ensured in every step of the study. **Results:** Among the 45 cases, the age range were 20-67 years (mean age was 46.42 years; SD 11.80). Majority of the tumors (n = 23, 51.11%) were diagnosed as serous carcinomas, 12 (26.67%) cases were diagnosed as endometrioid carcinomas, 10 (22.22 %) cases were diagnosed as mucinous cystadenocarcinomas. Seventeen (37.8%) cases were low grade and twenty-eight (62.3%) cases were high grade. Among the 23 serous carcinomas ,14 cases were high grade and 9 cases were low grade. Ten cases were diagnosed as mucinous cystadenocarcinomas and out of the them two cases were low grade and eight cases were high grade carcinoma. In case of endometrioid carcinomas, six cases were low grade and six cases were high grade. p16 score among 45 patients, 11 (24.4%) cases were negative and 34 (75.6%) cases were positive. Statistically significant association was found between tumor grades and p16 expression ($p<0.05$). **Conclusion:** This study found statistically significant association between histopathological grade and p16 expression ($p<0.05$). These findings may be helpful for selecting appropriate therapy for ovarian carcinoma patients.

KEYWORDS: Malignant ovarian, malignancy, carcinoma patients, serous carcinomas, p16.

INTRODUCTION

After cervical and uterine cancer ovarian cancer is one of the most common gynecological cancer which ranked 3rd.[1] In term of incidence India has the 2nd highest incidence of ovarian cancer & ovarian cancer is the seventh most common cancer among women worldwide.[2] Ovarian neoplasm has become increasingly important due to increased mortality rate.[3] It accounts for 2.5% of all malignancies among females but 5% of female cancer deaths because of low survival rates which indicate how deadly the tumor is.[4]

Ovarian tumors are composed of large variety of tumor which arise from epithelial cells, germ cells and stromal cells. Most of the ovarian malignancy are epithelial in origin (95%).[5] Predominantly in pre-menopausal and perimenopausal women suffer from ovarian malignancy, incidence of malignant tumors increases with age.[6] p16

is a negative cell cycle regulator encoded by the INK4a/ CDKN2A gene. It binds to cyclin-dependent kinases (CDKs) CDK4 and CDK6 inhibits the phosphorylation of RB resulting in cell cycle arrest and suppressing cellular proliferation. The expression of p16 is down regulated in a large number of tumors and overexpression has also been described in several tumors.[7] In the case of ovarian cancer, p16 expression is most commonly altered due to promoter methylation and less commonly by homozygous deletion or mutation. In an attempt to maintain the control over cellular proliferation overexpression occurred which causes accumulation of inactive mutant proteins.[8]

Among the epithelial types, serous carcinoma showed highest expression of p16. Tumor which overexpressed p16 are highly aggressive and no targeted interventions are available**.** Few recent studies showed that this tumor can be treated with suicide gene therapy but that is under trial now.[9]

The p16–CDK4–cyclin D–Rb pathway abnormality occur in the majority of cancers and it is a target pathway for anticancer therapy. PD-0332991(Palbociclib) is a selective inhibitor of the CDK4/6 kinases causes cell cycle arrest & can enhance the effects of chemotherapy.[10] Ribociclib is another CDK inhibitor which is a promising therapeutic option for ovarian cancer treatment.[11]

The present study was intended to observe p16 expression in paraffin embedded tissue taken from surface epithelial ovarian carcinoma and its association with histopathological grading.

OBJECTIVE

General Objective

To evaluate the p16 protein expression in surface epithelial ovarian carcinoma and its association with histopathological grading.

Specific Objectives

- To determine the histopathological grading of surface epithelial ovarian carcinoma.
- To evaluate the p16 protein expression in surface epithelial ovarian carcinoma.
- To assess the association of p16 protein expression with histopathological grade.

MATERIAL AND METHODS

Type of the study: Cross sectional observational study.

Place of the study: Department of Pathology, Chittagong Medical College, Chattogram, Bangladesh. Immunohistochemical study at Bangabandhu Sheikh Mujib Medical University, Dhaka.

Study period: March 2019 to January 2021.

Study sample

Total 45 histologically diagnosed cases of surface epithelial ovarian carcinoma was included in this study. The cases were taken from already excised and histologically reported tumour specimens at the department of pathology, Chittagong medical college. Some cases were collected from private laboratories of the Chattogram city.

Sample size

The aim of sample size calculation is to estimate the population prevalence with a good precision**.** In Bangladesh, there was no cancer registry of surface epithelial ovarian carcinoma. As no exact prevalence data was available, so assumed prevalence was 50%. But due to COVID-19 pandemic situation and unavailability of patients in the mentioned time, only **45** cases were collected.

Selection Criteria

Inclusion criteria

1) Patient histopathologically diagnosed as surface epithelial ovarian carcinoma.
2) Those patients who had given informed written consent for the study.

Exclusion criteria

1) Patients previously treated with chemotherapy or radiotherapy.
2) Histopathologically diagnosed non epithelial ovarian tumor.
3) Those patients who did not give informed written consent for the study.

Data collection tool

A predesigned Case record form.

Data collection procedure

Data was recorded on variables of interest by interview and using the structured questionnaire after taking properly informed written consent from the patient at the Department of Pathology, Chittagong Medical College. Patients were eligible for inclusion if they were undergone surgical resection for ovarian carcinoma and all the specimens of each case were submitted for histological examination; and finally diagnosed as surface epithelial ovarian carcinoma according to the WHO classification 2020. Two Tier Grading System was used to evaluate the grading of surface epithelial ovarian carcinoma.

Histopathologic Examination

All specimens of each case were processed by conventional histopathology method Hematoxylin and eosin-stained slides of each case was prepared for proper microscopic evaluation of tumor type, grade and invasiveness. Each slide was examined by at least two pathologists.

Immunohistochemical Examination

From paraffin-embedded blocks, 5-micrometer thick sections were cut, deparaffinized with xylene and rehydrated through a graded series of alcohol. For antigen retrieval, the samples were treated with Dako Target Retrieval solution (pH 9.0 for P16). Solutions were taken in coplin jar and pre-heated in the water bath at 65◦C. Then slides kept in this solution and heated in the water bath at 95-99◦C for 30-40 minutes. Then the sections were stained successively with Rabbit monoclonal p16INK4a antibody (Dako). Immunostaining was done manually following the avidin-biotin-peroxidase staining method. For p16 immunostaining, positive control was taken from section of invasive squamous cell carcinoma of cervix.

Evaluation of p16 Status

In this study, German semiquantitative scoring system was adopted. Interpreted the cytoplasmic and nuclear staining separately, as well as a mix-up of cytoplasmic and nuclear staining. German semiquantitative scoring system had been widely accepted and used in varies studies. Every tumor was given a score according to the intensity of the nuclear or cytoplasmic staining and percentage of stained cell. The final immunoreactive score was determined by multiplying the intensity and extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 12.

Statistical analysis

Data were entered into Excel worksheet to generate a master sheet. Then they were fed into software Statistical Package for the Social Sciences, version 27 (SPSS Inc., Chicago, IL) for processing and analysis. Qualitative variables (parity, menstrual history, histological type, histopathological grade, contraceptive history, p16 expression were expressed as frequency and percentage. Continuous variable (age of patient) was expressed as frequency, percentage, mean ±SD, mode, mean and range.

RESULTS

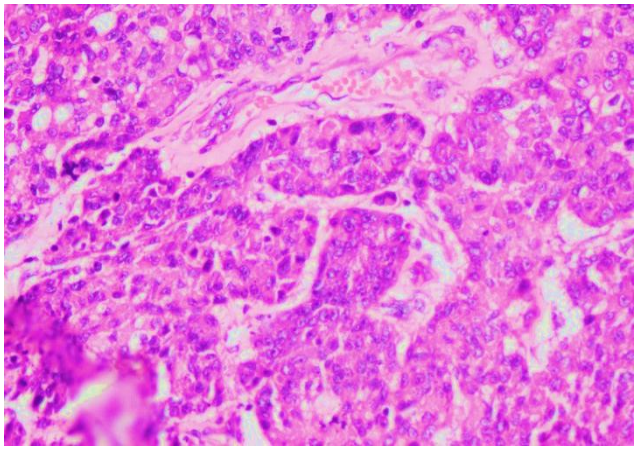


Fig 01: Serous carcinoma, grade II H & E (20x)

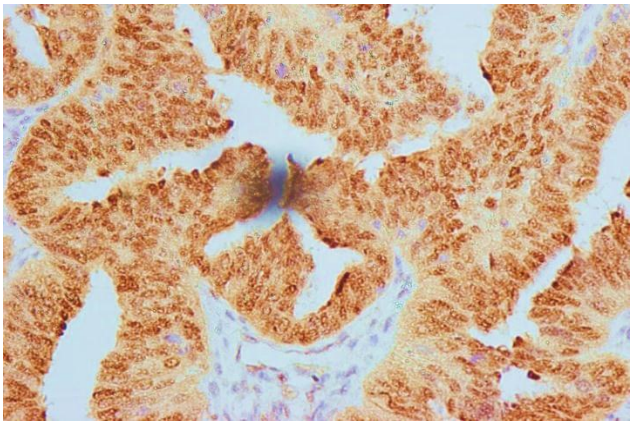


Fig 02: Serous carcinoma, grade II, (x40) p16 expression positive (nucleus & cytoplasm)

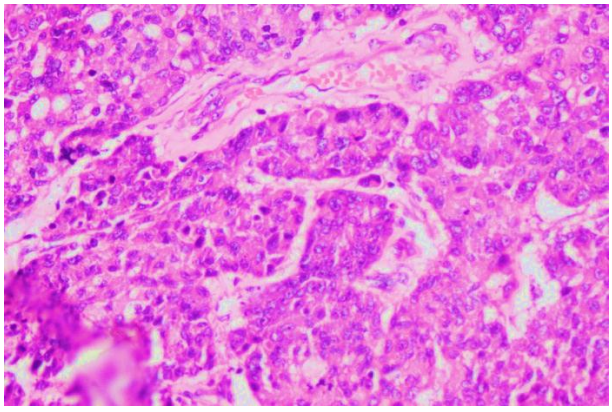


Fig 03: Serous carcinoma, grade III H & E (40x)

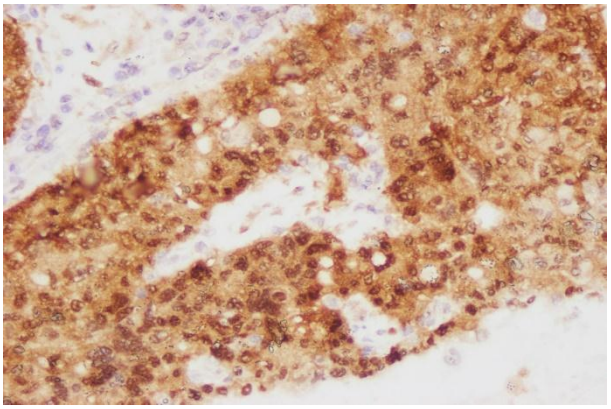


Fig 04: Serous carcinoma, grade III, (x40), p16 expression positive (nucleus & cytoplasm)

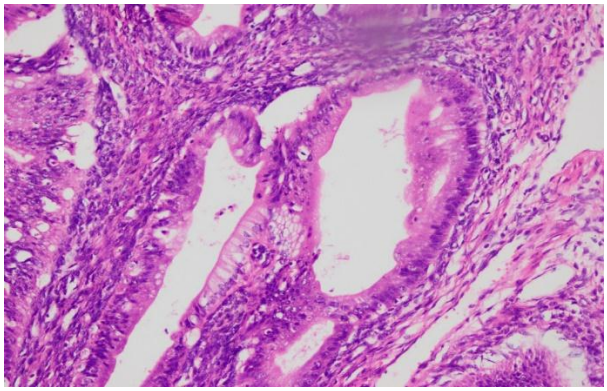


Fig 05: Mucinous adenocarcinoma, grade II H & E (20x)

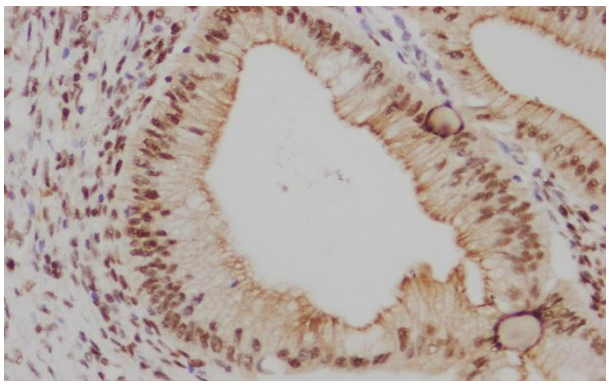


Fig 06: Mucinous adenocarcinoma, grade II (x40) p16 expression positive (nucleus & cytoplasm)

Distribution of the patients according to age (n=45):
Maximum (n=14,31.1%) patients were in between 51-60 years of age. Mean age (±SD) of the patients was 46.42 ± 11.80 years. Minimum age was 20 years and maximum age was 67 years (Table 1)

Table 1: Distribution of the patients according to age (n=45).

Age (years)	Frequency	Percent
≤30	5	11.1
31-40	10	22.2
41-50	14	31.1
51-60	10	22.2
>60	6	13.3
Mean ± SD (Min-Max)	46.42 ± 11.80 (20-67)	
Median	47	
Mode	49	

Distribution of the patients according to menstrual cycle (n=45)
In this study, thirty-six (80%) patients had history of regular menstrual cycle and nine (20%) patients had history of irregular menstrual cycle. (Figure 1).

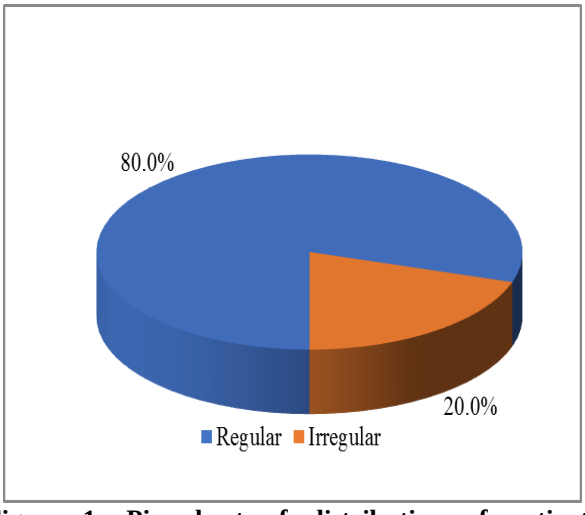


Figure 1: Pie chart of distribution of patients according to menstrual cycle (n=45)

Distribution of the patients according to parity (n=45)
Among parity distribution, most of the cases (n=36, 80.0%) were multiparous, 5 (11.1%) cases were nulliparous, 4 (8.9%) cases were primiparous. (Table 2).

Table 2: Distribution of the patients according to parity (n=45)

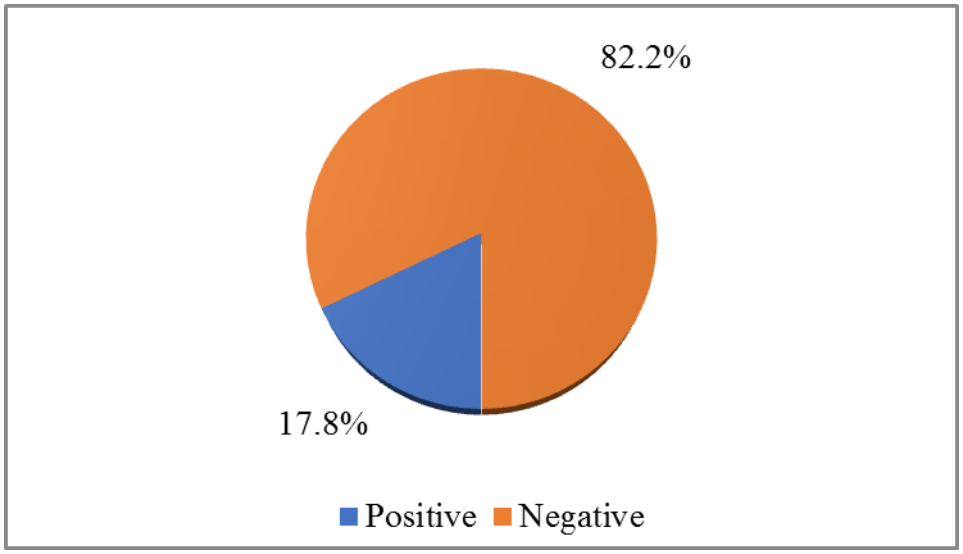
Parity	Frequency	Percent
Nulliparous	5	11.1
Primiparous	4	8.9
Multiparous	36	80.0
Total	**45**	**100.0**

Distribution of the patients according to contraceptive history (n=45)
In this study, it was seen 14 (31.1%) patients had history of hormonal contraception, 29(64.4%) patients had no history of contraception, only two (4.4%) cases had history of using barrier method (Table 3)

Table 3: Distribution of the patients according to contraceptive history (n=45)

Contraceptive history	Frequency	Percent
No contraceptive history	29	64.4
Hormonal	14	31.1
Barrier	2	4.4
Total	**45**	**100.0**

Distribution of the patients according to HRT (n=45)
In the present study, maximum (n=37, 82.2%) patients had no history of hormone replacement therapy (HRT) and 8(17.8%) patients had history of HRT (Figure 2)



Distribution of the patients according to histopathological diagnosis (n=45)

In this study, 23 (51.11%) cases were histologically diagnosed as serous carcinoma, 12(26.67%) cases were diagnosed as endometrioid adenocarcinoma and ten (22.22%) cases were diagnosed as mucinous adenocarcinoma (Table 4).

Table 4: Distribution of the patients according to histopathological type(n=45)

Histopathological diagnosis	Frequency	Percent
Mucinous adenocarcinoma	10	22.22
Serous carcinoma	23	51.11
Endometrioid adenocarcinoma	12	26.67
Total	**45**	**100.0**

Distribution of patients according to histological type and grade (n=45)

Among the 45 cases, twenty-three cases were serous carcinoma, nine cases were low grade and fourteen cases were high grade. In case of endometrioid carcinoma six cases were low grade and six cases were high grade. Ten cases were mucinous carcinoma and out of the them two cases were low grade and eight cases were high grade. (Table 5).

Table 5: Distribution of patients according to histological type and grade(n=45)

Grade	Serous carcinoma	Endometrioid adenocarcinoma	Mucinous adenocarcinoma
Low grade	09(39.13%)	06(50%)	02(20%)
High grade	14(60.87%)	06 (50%)	08(80%)
Total	23	12	10

Association of p16 expression with tumor grade

In the present study, tumor was graded according to two tier grading system into low grade and high grade. Statistically significant association was found in between histopathological grade and p16 expression ($p<0.05$).

Table 6: Association p16 with histopathological grades (n=45)

Histopathological grade	p16 expression		p value*
	Positive	Negative	
Low grade	9 (52.9)	8 (47.1)	0.011*
High grade	25 (88.0)	3 (12.0)	
Total	34	11	

*** p <0.05 is considered significant.**

*Fisher's Exact test was done to measure the level of significance.

Figure within parenthesis indicates in percentage.

Distribution of p16 expression according to tumor type: Table 7

Tumor type	p16 positive	p16 negative
Serous carcinoma	19	04
Mucinous carcinoma	07	03
Endometrioid carcinoma	08	04

DISCUSSION

Ovarian cancer is a salient public health concern, representing the 7th most common form of cancer and the 8th leading cause of cancer-related death among women worldwide. Its incidence is rapidly rising in East Asia. It is the deadliest form of gynaecological malignancy.[12] Unfortunately, the disease is not very symptomatic and has a low survival rate because a widely screening test has not yet been developed.

In the present study, mean age of the patients was 46.42 ± 11.80 years with a range from 20 to 67 years. This finding was nearly similar to the study of Farooq *et al.* (2013), who found mean age of ovarian cancer patient was 43.6 years (range: 15-70 years).[13] Agrawal *et al.* (2015) showed malignant tumours were more common after 40 years of age.[14] Another study by Ahmed *et al*., (2018) at BIRDEM hospital found mean age of patient with ovarian cancer was 47.5 years (range: 20–50 years) which was also nearly similar to this study.[15]

Among parity distribution, five (11.1%) cases were nulliparous, four (8.9%) cases were primiparous and thirty-six (80.0%) cases were multiparous. Study done by Bordelon *et al.* (2013) showed 16.1 % cases were nulliparous, 71.8 % cases were multiparous and 12.1% cases were primiparous. Both studies showed ovarian cancers were more common among multiparous.[16] Pregnancy causes anovulation and reduce the risk of ovarian cancer. Parous women have a 30%-60% lower risk than nulliparous women and each additional full-term pregnancy lowers risk by approximately 15%.

In the present study, 14 (31.1%) patients had history of hormonal contraception, 29 (64.4%) patients had no history of contraception, only 2 (4.4%) patients had history of using barrier method. According to Tsilidis *et al.* (2011) 58.9 % patients had history of hormonal contraception and 40.6% had no history of use of hormonal contraception.[17] This study was not similar to our study. In the present study, most of the patients were from low socioeconomic background, they were not much educated about contraceptive method, so most of the patients did not use any contraceptive method.

In the present study, 82.2% patients had no history of hormone replacement therapy (HRT) and 17.8% patients had history of HRT.

In present study, 23(51.11%) cases were histologically diagnosed as serous carcinoma, 12 (26.67%) were diagnosed as endometrioid adenocarcinoma, 10 (22.22 %) cases were diagnosed as mucinous adenocarcinoma. Study done by Vaidya *et al.* (2014) and Ahmad *et al.* (2000) were found serous carcinoma was most prevalent which was similar to our study.[18,19] But Sharma *et al.* (2000) found endometrioid adenocarcinoma was most prevalent.[20] The proportion of ovarian carcinoma subtypes does vary by country. The most substantial deviation was seen in the Asia/Oceania region, with Singapore, Japan, and Thailand having a smaller proportion of serous carcinomas, and larger proportions of endometrioid, clear cell, and mucinous carcinomas.[21] Some other variants of ovarian carcinoma were not found during this study period, which may be due to small sample size and limited period of time**.**

In the present study among 45 cases, seventeen (37.8%) cases were low grade and twenty-eight (62.3%) cases were high grade. In this study, among the 23 serous carcinomas 14 cases were high grade and 9 cases were low grade. Study done by Neill *et al.* (2007) found 22 low-grade ovarian serous carcinoma and 24 high-grade ovarian serous carcinomas.[22] Study done by Sallum *et al.* (2018) which was conducted on 106 serous ovarian carcinoma cases and sample was composed of 85 cases of HGSOCs and 21 cases of LGSOCs.[23] In case of ten mucinous adenocarcinoma two cases were low grade and eight cases were high grade carcinoma. In case of endometrioid adenocarcinoma six cases were low grade and six cases were high grade carcinoma.

Immunostaining score of 0–3 considered negative and 4 - 12 considered positive stain.[24] The scoring was done by using the 40x objective lens and counting at least 100 cells for immunoreactivity in 10 fields.[25] In the present study, tumor was graded according to two tier grading system into low grade and high grade.[26] In the present study, 34 (75.6%) cases were p16 positive and 11 (24.4%) cases were p16 negative. Study done by Neill *et al.* (2007) found statistically significant difference in p16 expression in between high-grade serous carcinoma and low-grade serous carcinoma.[22] High-grade serous carcinomas showed 88% positive tumour cells but some low-grade tumor also showed p16 positivity. In the present study also found some low-grade tumor expressed p16 positivity. In another study done by Ferguson *et al.* (2015) showed p16 positivity was more in high grade serous carcinoma than low grade serous carcinoma.[27]

In relation to epithelial types, serous tumors showed highest expression, while mucinous and endometrioid tumors demonstrate low levels or absent staining.[28] In this study,7 cases of mucinous adenocarcinomas showed p16 positivity and 3 cases showed p16 negativity. Some metastatic mucinous carcinoma may have included as primary mucinous carcinoma. Those could not be

excluded due to lack of proper history and radiological evaluation. As primary mucinous tumor is difficult to distinguish from metastatic carcinoma without proper history. A study done by LAM *et al* (2008) showed colorectal mucinous carcinoma showed p16 positivity.[29] So, may be some of the metastatic tumors were showing p16 positivity. Primary ovarian mucinous tumors are difficult to distinguish from metastatic mucinous tumors from the appendix, colon/rectum, cervix, or pancreas.[30]

In another study done by Lim *et al.,* (2016) revealed among the 31 endometrioid adenocarcinomas only 3(11%) cases were p16 positive rest of them were negative.[31] In present study, among the 12 endometrioid adenocarcinomas 8(66.67%) cases were p16 positive and 4(33.33%) cases were p16 negative. Which was not similar to our study.

CONCLUSION

In this study, it was observed that the proportion of tumors positive for p16 expression was higher among patients with high grade ovarian carcinoma. Statistically significant differences were found in tumor grade with p16 overexpression.

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