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RESEARCH ARTICLE

Immunohistochemical Protein Tracing of p15 INK4B Gene Expression in Latent Membrane Protein-1-Epetein Barr Virus Oncoprotein Infected Tissues from Ovarian Tumors Patients

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Abstract

This study was designed as a retrospective case-control study research. A total number of (150) formalin-fixed, paraffin embedded ovarian tissues was included. These tissues were including 45 malignant ovarian tumors (which including Serous epithelial (32 cases : 71.11%), Mucinous (9 cases :20%) and Endometrium (4 cases :8.89 %) , 45 benign ovarian tumors), 20 borderline tumors and 40 of apparently healthy ovarian sections which were included as baseline control .The mean age of the patients with ovarian carcinoma was higher (42.58±8.083years) than the mean age of the borderline ovarian tumor (41.90±10.126) and benign tumors group (39.82± 7.646 years), followed by mean age of those females in the group of apparently healthy control (35.64± 10.183). The results of immunohistochemistry staining of *LMP1-EBV* gene expression showed that 57.8% (26 out of 45 tissues) was positive in malignant ovarian tumors .While, in benign ovarian tumors the positive results was 40 % (18 out of 45 tissues) of *LMP1*-EBV. Whereas**, the** positive results in borderline ovarian tumor group was 40% (8 out of 20 tissues)**,** followed by the apparently healthy ovarian control tissues was 15% (6 out of 40 tissues)**.**

Introduction

Ovarian tumors occur as either an epithelial or a non-epithelial tumor. Epithelial ovarian cancer (EOC), also called ovarian carcinoma, typically begins in the epithelial cells on the surface of an ovary, which holds a proportion of 85% to 90% in ovarian cancers [2]. In Iraq, ovarian cancer is the fifth most common cause of death, and the 6th in the list of most common cancers.

Since most ovarian cancer patients are diagnosed at late stages, the overall survival rate remains a dismal 30% [3]. The incidence of ovarian cancer is higher in white women compared to African-American women, but the African American women have a more poor survival and higher mortality. The racial disparities have increased over time, partly due to differences in treatment, such as receipt of surgery [29].

Several risk factors which may contribute to the incidence of ovarian cancer have been high-lightened and the risk factors for different subtypes of ovarian cancer may differ from each other, especially among the EOCs such as Age of Woman; Reproductive history; Gynecologic surgery; Hormones; Fertility and Infertility Drugs; Hereditary; Smoking And Alcohol Use; Diets and viruses [28]. The Epstein-Barr virus encoded LMP1 oncoprotein modulates cell adhesion via regulation of activin A/TGFß and ß1 integrin signaling [29]. In last years, evidence has emerged which indicates that Herpes virus may also have a role in ovarian cancer. Previous studies have shown evidence of *Epstein–Barr-virus* in ovarian cancer [1].

LMP1 is a 66-kDa integral membrane protein that shares signaling properties with members of the TNF receptor superfamily. LMP1 has been shown to engage the three classic mitogen-activated protein kinases (MAPKs): ERK-MAPK, p38 MAPK and JNK/SAPK, the canonical and non-canonical NF-κB pathways, and the PI3K pathway [5].

LMP1 behaves as a classical oncogene, transforming rodent fibroblasts in vitro and rendering them tumourigenic in vivo [4]. EBV-mediated growth transformation is characterized by the expression of a subset of viral gene products, including latent

membrane protein- 1 (LMP-1) and LMP-2 A/B, as well as the nuclear proteins EBNA-1, -2, -3A, -3B, and -3C and LP [6].

These proteins coordinately regulate host signaling pathways to drive resting B cells to proliferate and ensure cell survival by inducing strong anti-apoptotic signals [7]. The p15 ^{INK4B} gene encompasses 6.41 kb of DNA and has 2 coding exons. It is tandemly linked to p16 INK4A and p14 ARF within 42 kb of genomic locus located on chromosome 9 p21. The locus is commonly referred to as INK4\ ARF locus [9].

One of the negative regulators of the cell cycle is p15 INK 4B, which binds CDK4, preventing the formation of an active CDK4-cycline complex. CDK- inhibitors, which are a new class of small proteins involved in the negative regulation of cell cycle, have been identified by virtue of their ability to interact physically with cycline\CDK- complexes.

As the gene, encoding the CdK4- and CDK-6 inhibitors (CDK4\6- inhibitors).P15$^{INK4B\MTS2}$ have been found to be altered in many cancer cell lines and primary neoplastic tissues, CDK- inhibitors in general and CDK4\6-inhibitors in particular are now a set of candidate tumor suppressors [9].

For the limited scope of this article, it was addressed this research work for the risk rates of both Lmp1-EBV and the expression of P15 gene in relation to grade and types of a group of tissues from Iraqi female patients with ovarian tumors.

Materials and Methods

Study Groups

This retrospective study was comprising collective number of (150) paraffin embedded ovarian tissue blocks enrolled from both patients and control samples that their age ranged from 19 to72.

They included (45) biopsies from patients who had undergone surgical biopsies from their ovarian cancers ;(45) biopsies from benign ovarian tumors; [20] biopsies from their borderline ovarian tumors and (40) biopsies from their apparently healthy ovarian tissues.

Laboratory Methods

A. Slide Preparation

Tissue sectioning was conducted at the histopathological department of Teaching laboratories / Medical City where one 4 mm-thick tissue section was stained with hematoxyline and eosin while another 4 mm-thick-tissue section were stuck onto positively-charged slides to be used for detection of –LMP-1-EBV by using specific mouse and rabbit- HRP/DAB (ABC) IHC kit (purchased from Abcam, UK) as well as using specific rabbit monoclonal primary Anti-p15 antibodies purchased from (Abcam, UK).

The methods for performing each specific-IHC reactions were conducted according the instructions of the manufacturing company and were done in the Advance Microbiology Laboratory / Virology Unit, at College of Science, University of Babylon.

B.Histopathological Analysis

The proper IHC detection system gives an intense brown signal at the specific positive sites of the examined tissues. The cells showing positive signal were evaluated under light microscopy at × 100 lenses in 10 different fields of 100 cells for each sample and the IHC results were given intensity and percentage scores based on intensity of signals as well as the number of cells gave signals, respectively.

A scale was used for relative intensities from 0 for no detectable IHC reaction, through 1, 2, 3 for low, moderate, and high intensity, respectively, and reactions assigned to one percentage of scores: 1%–25% as score 1, 26%-50% as score 2 and > 50% as score 3.

Statistical Analysis

ANOVA test and Chi square were used for statistical analysis of results by using the SPSS program and Excel application.

Results

Histological Types of Malignant Ovarian Tumors**:**

Frequency distribution of histological types according to biological behavior was Serous epithelial (71.11%), Mucinous (20%) and Endometrium (8.89 %).There were significant statistical differences ($p<0.05$) between malignant ovarian tumors group according to histological types (Table 1).

Table 1: Distribution of malignant ovarian tumors group according to histological types

Ovarian Cancers		Total (N=45)	%	P-value
Types	Endometrium	4	8.89%	χ^2 test P=0.004 sign. (P>0.05)
	Mucinous	9	20%	
	Serous	32	71.11%	

Histopathlogical Grades of Malignant Ovarian Tumors

The results of present study show that moderately grades ovarian carcinomas (grade II) constituted 35.5% (16 out of 45 tissues) , whereas with poorly differentiated grade ovarian carcinomas (grade III) constituted 33.3% (15 out of 45 tissues) and well differentiated (grade I) 31.2% (14 out of 45tissues) , respectively. The results reveal non-significant differences at (P>0.05) between poorly differentiated grade and well differentiated grade, also non-significant difference was noticed between poorly differentiated and moderately differentiated ovarian carcinomas (Table 2) .

Table 2: Grading of ovarian cancers group

Ovarian Cancers		Total (N=45)	%	P-value
Grades	I	14	31.2	χ^2 test P=0.843 Non sign. (P>0.05)
	II	16	35.5	
	III	15	33.3	

Histological Types of Benign Ovarian Tumors

The most common histological type from benign ovarian tumor study cases was cystic teratomas including 34/45 (75.56%) cases, followed by the fibromas 11/45 (24.44%) .There were highly significant statistical differences (p<0.01) between different groups according to age (Table 3).

Table 3: Distribution of malignant ovarian tumors group according to histological types

Benign ovarian tumor		N (Total N=45)	%	P-value
Type	Cystic teratomas	34	75.56%	Z test P=0.001 Highly sign. (P<0.01)
	Fibromas	11	24.44%	

Age Distribution among Study Groups

The patient's ages ranged from 19-72 years with a mean of 39.985 years .The mean age of patients with malignant ovarian tumors (42.58 years) was higher than the mean age of the borderline tumors & benign ovarian tumors (41.90 years) and (39.82 years), respectively . While, the mean age of apparently healthy control(AHC) was (35.64 years). However, there was highly significant differences at (p<0.01) between different groups in age distribution (Table 4).

Table 4: Distribution of ovarian Tumors patients according to their age

Studied groups	N	Mean (Age / Year)	Std. Deviation	Std. Error	Range		ANOVA Test (P-value)
					Mini.	Maxi.	
A.H. Control	40	35.64	10.183	1.610	27	60	P=0.003 Highly sign. (P<0.01)
Benign tumor	45	39.82	7.646	1.140	19	55	
Border line tumor	20	41.90	10.126	3.202	29	57	
Ovarian cancer	45	42.58	8.083	1.205	25	72	
Total	150	39.985					

The IHC-Evaluation of Latent Membrane Protein- 1- EBV (LMP1) in Ovarian Tumors

The results showed that 57.8% (26 out of 45 tissues) of *LMP1-EBV* - gene expression was positive in malignant ovarian tumors .While, in benign ovarian tumors the positive results was 40 % (18 out of 45 tissues) of *LMP1*-EBV**.** Whereas ,the positive results in borderline ovarian tumor group was 40% (8 out of 20 tissues)**,** followed by the apparently healthy ovarian control tissues was 15% (6 out of 40 tissues).Statistically, there was highly significant difference among them at (P<0.05) and as shown in (Table 5 & Figure 1).

Table 5: The Results of *LMP1-EBV*-IHC Gene Expression in Ovarian Tumors

LMP1-EBV		Studied groups				Pearson Chi-Square (P-value)
		A.H. Control	Benign tumor	Border line tumor	Ovarian cancer	
Positive	N	6	18	8	26	P=0.00 Highly sign. (P<0.01)
	%	15%	40%	40%	57.8%	
Negative	N	34	27	12	19	
	%	85%	60%	60%	42.2%	
Total	N	40	45	20	45	
	%	100%	100%	100%	100%	
Z test			P=0.233 NS	P=0.754 NS	P=0.023 S	

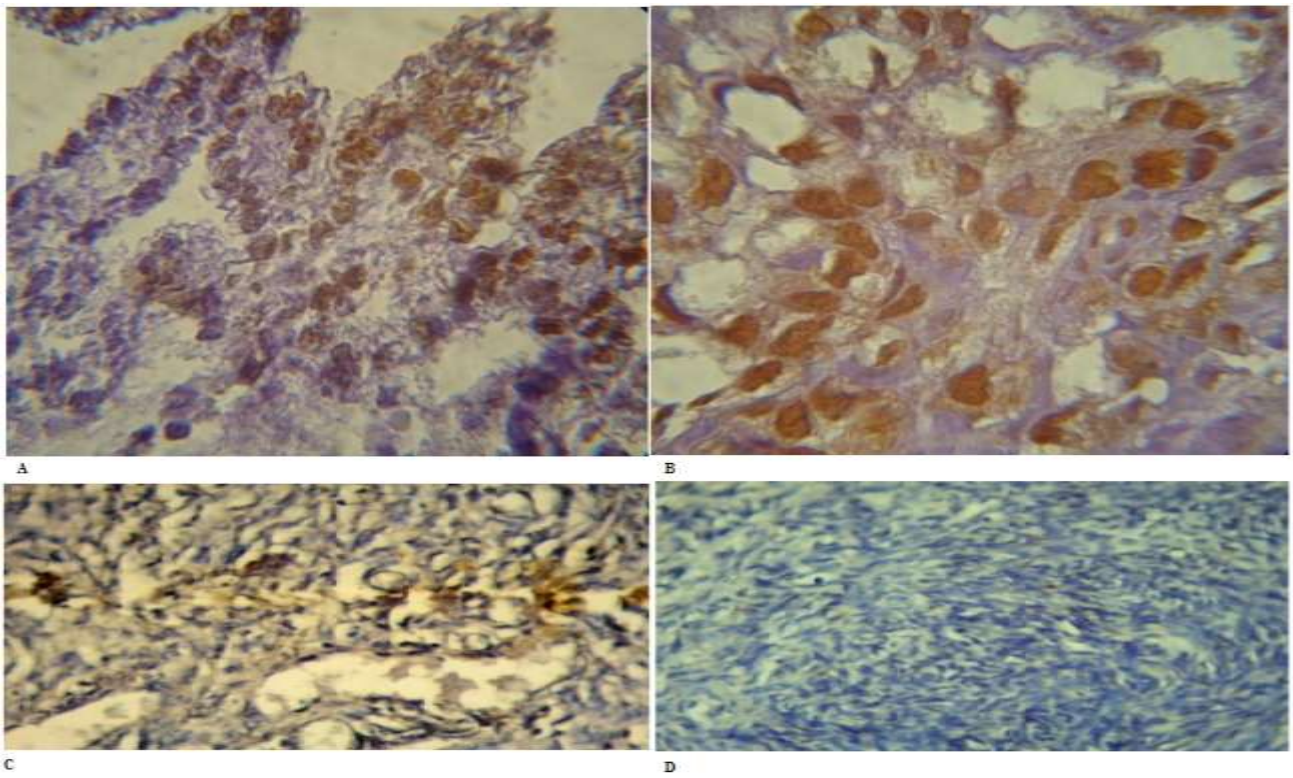

Figure 1: Microscopic appearance shows over expression of *LMP-1-EBV* protein in surface epithelial ovarian tumors. Stained by DAB chromogen (brown) and counter stained with Mayer's heamatoxylin. A. Serous Ovarian Cancer *LMP-1-EBV*, high score and strong intensity (X400). B. Mucinous cystadenoma *LMP-1-EBV*, high score and strong intensity (X1000). C.Benign ovarian tumors *LMP-1-EBV*, moderate score and moderate signal intensity (X1000). D. Negative *LMP1-EBV* –IHC reactions (X 400).

The IHC-Evaluation of P15 in Ovarian Tumors Tissues

The results show that 35.6% (16 out of 45) of *p15-* protein expression were positive in ovarian cancer tissues. Whereas, in benign ovarian tumors the positive results was 37.8% (17 out of 45 tissues) of *P15* protein expression. While, in borderline ovarian tumors group was 50 % (10 out of 20 cases) followed by the apparently healthy ovarian control tissues was 5% (2 out of 40 tissues) .Statistical analysis of the *P15-* immunohistochemical assay tests show highly significant difference (p>0.01) Table (6) and Figure (2).

Table 6: Distribution of IHC-*P15* Expression Signals in Female Patients with Ovarian Tumors

P15		Studied groups				Pearson Chi-Square (P-value)
		A.H. Control	Benign tumor	Border line tumor	Ovarian cancer	
Positive	N	2	17	10	16	P=0.001 Highly sign. (P<0.01)
	%	5%	37.8%	50%	35.6%	
Negative	N	38	28	10	29	
	%	95%	62.2%	50%	64.4%	
Total	N	40	45	20	45	
	%	100%	100%	100%	100%	

Z test		**P=0.135 NS**	**P= 1 NS**	**P=0.072 NS**	

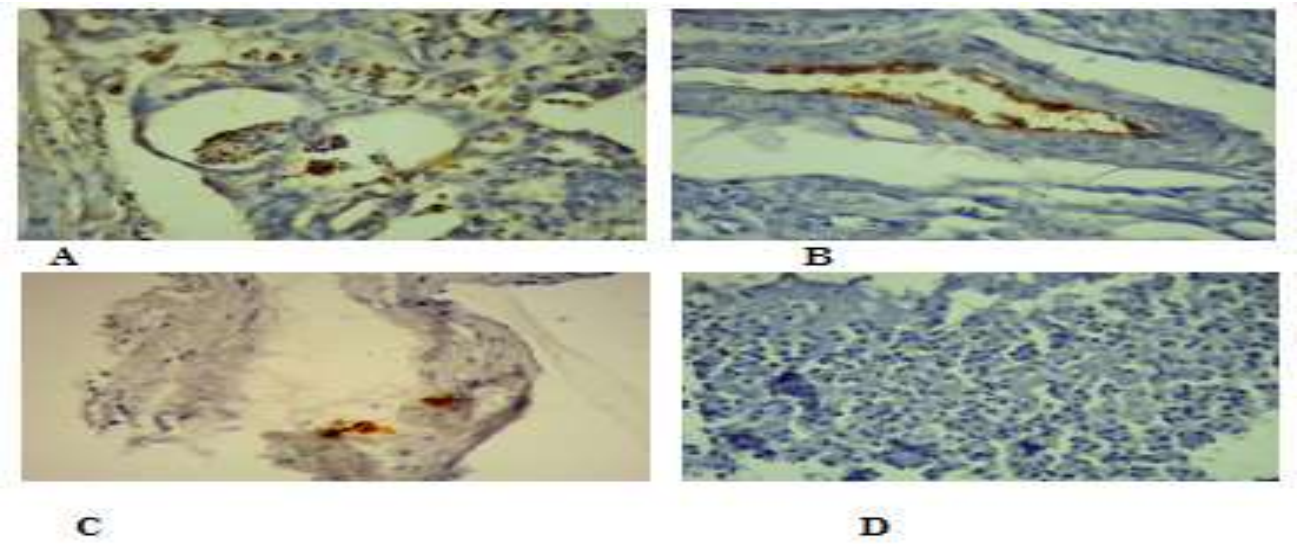

Figure 4-18: Microscopic appearance shows over expression of *P15* protein in ovarian tumors. Stained by DAB chromogen (brown) and counter stained with Mayer's heamatoxylin. A. Serous Ovarian Cancer, P15, high score & moderate intensity(X400). B.Mucinous Ovarian Cancer, P15, moderate score & moderate intensity(X1000). C. Benign Ovarian Tumors P15 reaction with low score and weak signal intensity (400X). D. Ovarian Tumors with Negative P15 reaction

Discussion

In Iraq, ovarian tumors rank the 6th commonest cancer, and it constituted 1.62%, 3.8%, and 4.18% in the years 1976-1978, 1992-1994, and 2001 (Ministry of Health result on Iraqi Cancer Registry1976-1978, 1992-1994 and 2001), while it was 7th commonest cancer and constituted 3.52% according to Iraqi Cancer Board in 2005 (Ministry of Health result on Iraqi Cancer Registry,2005). In 2004, in the United States, 50% of all ovarian carcinomas were bilateral. Malignant serous tumors constituted over 40% of invasive epithelial carcinomas. In the present study, was found the most common type the Serous epithelial (71.11%), followed by Mucinous (20%) and Endometrium (8.89 %) (Table 1) [10].

Found that patients who have invasive serous carcinomas usually acquire more aggressive biological behavior of ovarian carcinoma. Primary mucinous epithelial ovarian carcinoma (mEOC) is a relatively rare subset of epithelial ovarian cancers. The incidence of mucinous epithelial ovarian cancer is ~12% as exemplified by a recent population [11].

Endometriosis is a common gynecologic disorder. The estimated frequency among women of reproductive age is 5%–10% and is particularly frequent among women with pelvic pain and infertility.

In 1925, Sampson was first to describe the malignant transformation of endometriosis to ovarian carcinoma [12]. The results of present study show that poorly differentiated constituted 35.5% ,followed by moderately grades 33.3% and lastly , 31.2% for well differentiated. These results gave us an indication the old age women may be more susceptible to get malignancy for several reasons. Several factors related to these finding, the cell mediated immunity play an important role in the defenses against the cancer.

These results are compatible with Stanly report in 2005 who shows the importance of cellular immune responses in the resolution of viruses infection, it is not surprising that deficiencies in cell-mediated immunity increase the likelihood of disease expression (persistence or progression) in groups such as older women (waning immunity), transplant recipients, patients with HIV, and those receiving immunosuppressive drugs [11]. Studies on the prognostic implications of age and ovarian cancer are inconclusive.

Chan and his colleague when reported that, the distribution of tumor grade differed between young and old women. They found that in younger women with mean age 40±5.7 years, well, moderately, and poorly

differentiated carcinoma constituted 11%, 35%, and 54% respectively, compared to 4%, 11%, and 85% in older patients with a mean age of 61±8.7 [13].This results consistent with the study by Jaffar and Al-Alwany [14,15] On reviewing the 150 cases which were included in this study, it was found the age of the patients with ovarian tumors was ranging between 19-72 years and their mean age was 39.985 years .

The present results are consistent with those reported world-wide where these ovarian tumors were usually affecting females over forty years of age [16]. Ovarian cancer is a disease of elderly women. It has been speculated that age can affect prognosis in ovarian cancer: an independent but yet unexplained association of advanced age with prognosis, possibly due to different tumor biology; benign ovarian cysts are a common finding in women of all ages [17].

Also, it was noticed, the most commonly affected age of malignant Benign &Border line (41-60) years constituting, 53.3% 73.3% 60% .respectively .The present results could have their importance when realizing that the age of the ovarian cancer patients is an important factor both for the occurrence and management of the disease [18].

These results could reflect that age is an important risk factor in tumor changes affecting ovarian epithelial tissues lesions. In general, aging increase the incidence of the malignant changes in ovarian epithelial tissues and as such their incidence was found to increase with age [19]. Ovaries from older women have been noted to show more morphological changes than those from younger women [20].

Presumably ovulation and subsequent repair cause these age-dependent changes, or so-called ovarian ageing, which might represent preneoplastic areas or lesions [21]. Ovarian cancer is rare in women under the age of 40, reports the American Cancer Society (ACS). Fifty percent of all cases of ovarian cancer are found in women aged 63 or older. You're more likely to develop it after you reach menopause.

In fact, in this period 83% of the patients with ovarian cancer received some form of chemotherapy within 4 months of diagnosis progestin's may increase apoptosis in ovarian epithelium, which means women with a later age at menarche may have several extra years of low-level estrogen (or other hormone) stimulation of their ovarian epithelium in the absence of the apoptotic effects of progesterone, possibly increasing the chance of the cells acquiring genetic damage androgens [22].

EBV is among group-1 carcinogens as classified by IARC Working Group, However, the viral prevalence vary markedly with the associated cancers, as well as the virus differs in the patterns of expressed viral genes, suggesting that EBV may affect cell growth in more than one way [23]. Thus, EBV represents an important but not a sufficient step in carcinogenesis, and an additional epidemiological risk factors could play a critical role in this process.

The present results showed that 57.8% of *LMP1-EBV* - gene expression was positive in malignant ovarian tumors .While, in benign ovarian tumors the positive results was 40 % of *LMP1*-EBV**.** Whereas**, the** positive results in borderline ovarian tumor group was 40%**,** followed by the apparently healthy ovarian control tissues was 15% (6 out of 40 tissues). From these results LMP1, may be enhance the invasive and metastatic capabilities of EBV-infected malignant cells. However, our results are comparable to the results reported by [24] where ISH for LMP1 was detected in all the 87 biopsies (100%) in the nuclei of the tumor cells.

LMP1 was detected as a dot-like cell membrane and/or cytoplasmic staining signals in 55 out of 87 (63%) cases [25]. Was showed the high rate EBV LMP 1 tumor in malignant epithelial an EBV associated malignant disease, is unique in that it is an epithelial origin tumor and expresses EBV primary oncogene latent membrane protein 1 (LMP1) at the premalignant stage.

LMP1 is the only EBV-encoded protein with the characteristics of a classical oncogene, full understanding of the signaling capacity of LMP1 is crucial to defining its role in EBV-induced oncogenes is [19]. However, previous study has focused on the contribution of relation EBV LMP 1 with ovarian tumor according to results of present study.

Latent membrane protein-1 (LMP1), is likely responsible for many of the altered cellular growth properties in EBV-associated cancers.

In epithelial cells, LMP1 disrupts cellular adhesion through down-regulation of the intercellular adhesion protein E-cadherin and up-regulation of proteins involved in the disruption of the extracellular matrix [20]. Thus, LMP-1 EBV represents an important but not a sufficient step in carcinogenesis, and an additional epidemiological risk factors could play a critical role in this process.

The initial report by [22] showing that ovarian cell lines frequently (29%) had deletions at the 9p21 locus containing p1 suggested that one or more of these gene may play an important role in human ovarian tumor genesis. In the current study, positive p15 immunohistochemistry staining was detected in 35.6% of malignant ovarian tumors while in benign ovarian tumors were detected in 37.8%.

While, in borderline ovarian tumors group was 50 % followed by the apparently healthy ovarian control tissues was 5% .These findings could indicate for either loss of wild-type p15 function, gain of oncogenic function or the ability to activate p15 inappropriately that can severely compromised the capacity for controlled cellular proliferation and growth .

The results of this study are in agreement with other result revealed by [26] who examined the homozygous deletion of p15/MTS2 gene was found 15 (33%) cases. Also, Giuseppina D' *et al*., [27] who analyzed homozygous deletion and somatic mutation of p15/MTS2 genes, 49 primary ovarian tumors and 6 ovarian carcinoma cell lines. Homozygous deletion was found in 10% of primary tumors, but mutation of *p15* was not detected in any sample.

Alterations in *p15* were observed in serous, endometriosis, and clear cell carcinomas, but not in mucinous carcinomas, suggesting that inactivation of *p15* may be the histologic type-specific event in ovarian tumorigenesis he deletion of the gene was a potential indicator for poor chemotherapy response and a significant poor prognostic factor in advanced ovarian cancer. The p15 genes possess extensive sequence similarity, and localized on chromosome 9p21.

The p15 protein binds to and inhibits CDK function in vitro, and ectopic expression of p15 inhibits cell growth in vitro [28].Expression of p15 is independent of RB regulation, unlike the p16 gene. Not only the physiological functions but also their roles as tumor-suppressor genes seem to differ between the 2 genes. Point mutations in p16 are found in tumors with a varying frequency, depending on tumor type, but are extremely rare in the p15 gene [29].

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