Original Article

Detection of Human papillomavirus in surface epithelial ovarian carcinoma using in situ hybridization technique

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Summary:

Background: The role of Human papillomaviruses (HPV) in the etiology of ovarian cancer remains unclear and the results are controversial. Several studies have verified the presence of HPV DNA in both malignant and benign ovarian tumors.

Objectives: Determine the percentage of detection of HPV high (16&18) and low risk types (6&11) in surface epithelial ovarian carcinoma compared to benign and control groups.

Materials And Methods: Molecular detection and genotyping of HPV DNA were performed in 76 ovarian tissue blocks by using in situ hybridization (ISH) technique for detecting and localization of high risk HPV (16 and 18) and low risk HPV (6&11) types.

Results: The presence of ISH signals for HPV DNA in benign group (71%) was higher than that found in malignant group (64%). HPV 16 was the most predominant type followed by HPV18, 6, and 11 respectively in both malignant and benign groups. High risk HPV were presented with low score and high intensity in both malignant and benign tumors. Low risk HPV types were detected in high score and intensity in benign tumors which significantly differed from that with malignant tumors, which revealed low score and low intensity. The percentage of co-infection of low risk HPV6&11 in benign group was higher (16.9%) than malignant group (7.1%). Only significant difference was found in combination of both high and low risk HPV types.

Conclusions: This finding reflects a possible role of HPV virus in the carcinogenesis of ovarian tumors. HPV infection may play a relative role in the pathogenesis of ovarian carcinomas or it could facilitate its progression.

Key words: In situ hybridization, HPV, and ovarian carcinoma.

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Introduction:

Human papillomaviruses (HPVs) are small double-stranded DNA viruses featuring oncogenic properties. They infect mucosal and skin epithelia (1). Although human papillomaviruses (HPVs) have been associated with benign and malignant proliferative lesions of the genital tract (2), published reports of biopsy sample analyses have drawn conflicting conclusions on their potential role in ovarian cancer (3, 4, 5, 6, 7, & 8). Analysis of cervical cancer derived cell lines helped in defining the association between HPV and cervical cancer (2 & 9). Such an approach has not been reported for ovarian cancer. In this study, we investigated the presence of high and low risk HPV types in ovarian tissues using In Situ Hybridization technique.

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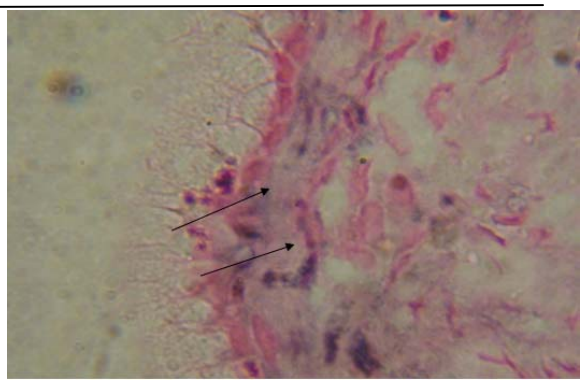
Subjects, materials and methods:

The study was designed as a retrospective one. It involved 76 selected formalin fixed, paraffin embedded ovarian tissue blocks including 28 blocks of surface epithelial ovarian carcinomas, 28 blocks of benign epithelial ovarian tumors, and 20 blocks of normal ovarian tissues as a control. The ages of their related cases range from 20-72 years. Specimens were collected during the period from June 2007 to June 2010 from the Medical City Teaching Hospital, and Al-Elweiya hospital. The diagnosis of these tissue blocks were based on the obtained pathological records of these cases from hospital files as well as histopathological laboratories records. A confirmatory histopathological re-examination of each obtained tissue blocks was done. Four µm thick sections were made and sticked on positively charged slides. In situ Hybridization /Detection system (Maxim Biotech Inc. USA) used to target DNA sequences using biotinylated long DNA probe for HPV16, 18, 6, and 11 in tissue specimens. Method was conducted according to the instructions of manufacturing company leaflet. Positive control reactions were

performed by replacing the probe with biotinylated house keeping gene probe. For the negative control, all reagents were added except the diluted probe. Proper use of this hybridization/detection system gave an intense blue signal at the specific site of the hybridization probe in positive test tissue. Quantification of different molecular markers in situ hybridization signal was evaluated under light microscopy and the counting of positive cells was performed at X1000. In situ hybridization was given intensity and percentage scores, based on intensity of positive signals and number of signals, respectively. The intensity score included low, moderate, and high intensity of reaction. Positive cells were counted in ten different fields of 100 cells for each sample and the average of positive cells of the ten fields was determined assigning cases to one of the three following percentage score categories: Score(1) = 1-25%, Score(2)= 26-50%, Score(3)>50% (10). Chi-square test was used to detect the significances between variables of our study. All the statistical analysis was done by SPSS program (version-17). P value was considered significant when < 0.05.

Results:

The presence of ISH signals for HPV DNA in benign group (71%) was higher than that found in malignant group (64%). HPV DNA of types (16, 18, 6, and 11) were found in malignant group as 11(39.3%), 10(35.7%), 6(21.4%) and 5(17.9%) respectively, in benign group they were 13(46.4%), 12(42.9%), 10(35.7%) and 7(25%) respectively, lastly in control group constituted 2(10%), 2(10%), 0(0%), and 0(0%) respectively. Significant association (P<0.05) of all genotypes of HPV except HPV11 was found with study groups (Table-1). Co-infection of HPV types in ovarian tissues are shown in (Table-2). Co-infection of high risk types (HPV16 and 18) were detected in 7 cases (25%) of both malignant and benign ovarian tumors, and it was seen in one case (4.3%) of control group. Co-infection of low risk types of HPV (6 and 11) were found to be higher 17.9% (5cases) in benign group while it was 7.1% (2cases) in malignant group. Co-infection of both high and low risk types of HPV were found to be higher in benign group 35.7% (10cases) than in the malignant group which was 14.3% (4cases). Statistically no significant difference (P>0.05) was found on comparing study groups for co-infection of high risk types or for low risk types. A significant difference (P<0.05) was found on comparing the different study groups for the presence of co-infection of both high and low risk types. Figure (1) shows the positive results of HPV DNA by ISH technique according to score and intensity.



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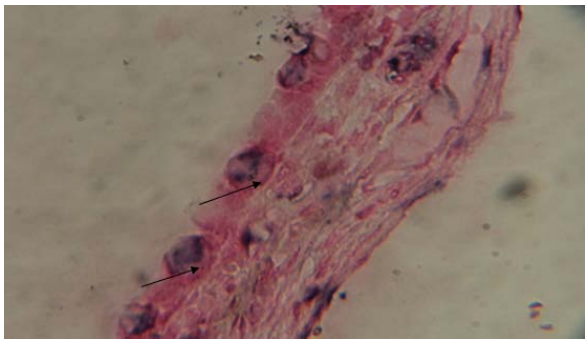

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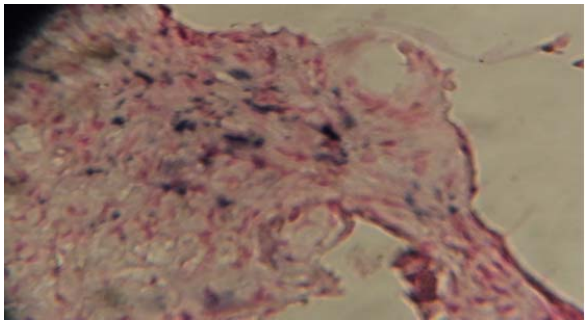

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Figure (1): Microscopic appearance of HPV-positive ISH signals of surface epithelial ovarian tumors. Blue signals are detected at complementarity sequences sites (arrows):
A) Mucinous cyst adenoma, HPV16 DNA, low score & low signal intensity(X1000)
B) Serous cyst adenoma, HPV18 DNA,low score & moderate signal intensity (X1000)
C) Serous cyst adenocarcinoma, HPV11 DNA, moderate score&high intensity (X400).

Detection Of HPV High Risk Types According To Score And Intensity:

Eleven cases of malignant group (39.3%) revealed positive signal for HPV16 DNA, benign tumors were positive in 13 cases (46.4%) and control group revealed positive signals in 2 (10%) of cases. A significant difference (P<0.05%) was found on comparing the percentages of HPV16 DNA among the study groups (Table-3). The positive cases of

malignant and benign groups for HPV16 DNA were higher in low score. They were 8 cases (28.6%) and 5 cases (17.9%) respectively. The malignant group showed high signal intensity in 5 cases (17.8%), low in 4 cases (14.3%) and moderate intensity in 2 cases (7.1%). In benign group high percentages of HPV16 were found in moderate intensity 6 cases (21.4%) followed by 5 cases (17.9%) and 2 cases (7.1%) in high and low signal intensity respectively. In control group there was only two cases positive for HPV 16, one of them found in moderate (5%) and the other one in high score (5%) while both of them showed high signal intensity (10%). Significant differences ($p<0.05$) were found on comparing the percentage of HPV16 DNA in study groups according to signal score and intensity. The positive result of HPV18 DNA in malignant, benign and control groups were 10 (35.5%), 12 (42.9%) and 2 cases (10%) respectively. As in HPV16 a significant difference ($P<0.05$) was found on comparing the results of HPV18 DNA among study groups. In the malignant group high percentage of HPV18 DNA was detected in low score 10 (35.7%) and high percentage was found in 4 cases (14.3%) of each of low and moderate signal intensity. For benign group high percentages of HPV18 DNA were detected in low score 7(25%) and in high signal intensity 6(21.4%). In control group 2 cases (10%) were detected in both high score and high signal intensity. Significant differences ($P<0.05$) were found between benign and malignant group on comparing the results according to signal score and intensity (Table -4).

Detection Of HPV Low Risk types According To Score And Intensity The positive results of HPV6 DNA in malignant, benign and control groups were 6 (21.4%), 10 (35.7%) and (0%) respectively. A significant difference ($P<0.05$) was found on comparing HPV6 positive results among study groups. In malignant group high percentages of HPV6 DNA were detected in low score 6 (21.4%) and low signal intensity 3 (10.7%) while in benign group high percentages of HPV6 DNA were found in high score 5 (17.9%) and high signal intensity 5 (17.9%). Significant difference ($P<0.05$) of HPV6 DNA was found on comparing the results according the score and signal intensity (Table -5).Positive results of HPV11 DNA in malignant, benign and control groups were (17.9%), (25%) and (0%) respectively. Significant differences ($P<0.05$) were found on comparing positive results among study groups (Table -6). In malignant group high percentages of HPV11 DNA were detected in low signal score (14.3%) and low intensity (10.7%), while in benign group high percentages of HPV11 DNA were found in high score (17.9%) and in both high and moderate signal intensity (10.7%). Significant differences ($P<0.05$) were found on comparing HPV11 results according to score.

Table (1): Frequency distribution of HPV DNA in ovarian tissues among study groups

Groups	Negative (%)	Total Positive (%)	Positive results in each HPV type*			
			HPV16	HPV18	HPV6	HPV11
Malignant	10/28 (36%)	18/28 (64%)	11 (39.3%)	10 (35.7%)	6(21.4%)	5 (17.9%)
Benign	8/28 (29%)	20 /28(71%)	13 (46.4%)	12 (42.9%)	10 (35.7%)	7 (25%)
Control	18/20(90%)	2/20(10%)	2 (10%)	2 (10%)	0 (0%)	0 (0%)
Chi square test Among groups			p<0.05, Sig.	p <0.05, Sig.	p < 0.05, Sig.	p>0.05,non Sig.

*** The total positive in the table refer to positive cases which may have more than one genotype of human papillomavirus and the positive results for each HPV genotype (16, 18 , 6, and 11) may also be positive for another genotype.**

Table (2): Co-infection of HPV types in ovarian tissue among study groups

		HPV Results			
Groups	Total	Negative	HPV(16+18)	HPV(6+11)	Low & high risk HPV
Malignant	28	10 (35.7%)	7 (25%)	2 (7.1%)	4 (14.3%)
Benign	28	8 (28.6%)	7 (25%)	5 (17.9%)	10 (35.7%)
Control	20	18 (90%)	1 (4.3%)	0 (0%)	0 (0%)
Chi square test			p>0.05, N.S.	p>0.05, N.S.	p<0.05, S

Table (3): Frequency distributions of positive HPV 16 DNA ISH signal score and intensity among study groups

Groups	Negative	Positive according to score			positive according to intensity			Total positive
		Score 1	Score 2	Score 3	Low	Moderate	High	
Malignant	17/28 60.7%	8/28 28.6%	1/28 4%	2/28 7%	4/28 14.3%	2/28 7.1%	5/28 17.8%	11/28 39.3%
Benign	15/28 53.6%	5/28 17.9%	4/28 14.3%	4/28 14.3%	2/28 7.1%	6/28 21.4%	5/28 17.9%	13/28 46.4%
Control	18/20 90%	0/20 .0%	1/20 5 %	1/20 5%	0/20 .0%	0/20 .0%	2/20 10 %	2/20 10%
Chi square test		Among study groups: p<0.05 , S.			According to score and intensity: P value <0.05, S.			

Table (4): Frequency distribution of in situ hybridization For HPV 18 DNA according to signal score and intensity among study groups

Groups	Negative	Positive results according to score			Positive results according to intensity			Total positive
		Score 1	Score 2	Score 3	Low	Moderate	High	
Malignant	18/28 64.3%	10/28 35.7%	0/28 0%	0/28 .0%	4/28 14.3%	4/28 14.3%	2/28 7%	10/28 35.7%
Benign	16/28 57.1%	7/28 25.0%	2/28 7.2%P	3/28 10.7%	5/28 17.9%	1/28 3.6%	6/28 21.4%	12/28 42.9%
Control	18/20 90%	0/20 .0%	0/20 .0%	2/20 10 %	0/20 .0%	0/20 .0%	2/20 10 %	2/20 10%
Chi square test	Among study groups: p<0.05 , S.				According to score and intensity: P value <0.05, S.			

Table (5): Frequency distribution of in situ hybridization for HPV 6 DNA according to signal score and intensity among study groups

Groups	Negative	Positive results according to score			Positive results according to intensity			Total positive
		Score 1	Score 2	Score 3	Low	Moderate	High	
Malignant	23/28 78.6%	6/28 21.4%	0/28 .0%	0/28 .0%	3/28 10.7%	2/28 7.1%	1/28 3.6%	6/28 21.4%
Benign	18/28 64.3%	2/28 7.1%	3/28 10.7%	5/28 17.9%	1/28 3.6%	4/28 14.3%	5/28 17.9%	10/28 35.7%
Control	20/20 100%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 .0%
Chi square test	Among study groups: p<0.05 , S.				According to score and intensity: P value <0.05, S.			

Table (6): Frequency distribution of in situ hybridization for HPV 11 DNA according to signal score and intensity among study groups

Groups	Negative	Positive results according to score			Positive results according to intensity			Total positive
		Score 1	Score 2	Score 3	Low	Moderate	High	
Malignant	23/28 82.1%	4/28 14.3%	1/28 3.6%	0/28 .0%	3/28 10.7%	1/28 3.6%	1/28 3.6%	5/28 17.9%
Benign	21/28 75.0%	2/28 7.1%	0/28 .0%	5/28 17.9%	1/28 3.6%	3/28 10.7%	3/28 10.7%	7/28 25%
Control	20/20 100%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 .0%	0/20 0%
Chi square test	Among groups: Significant (p < 0.05), According to score: P-value<0.05, Sig, According to intensity:P value > 0.05, NS.							

Discussion:

Ovarian cancer remains a highly lethal disease. In the United States, ovarian malignancy is a leading cause of cancer-related deaths among females and accounts for more deaths than all other gynecological neoplasm (11). 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 (12,13, & 14). Regarding the association of HPV with ovarian cancers up to our knowledge, this was the first study in Iraq which was designed to detect the association of HPV infection with surface epithelial ovarian tumors. For this purpose, the infection rates of HPV16, 18, 6, and 11 in ovarian surface epithelial ovarian tumors were analyzed. Generally speaking HPV DNA was found to be higher in benign tumors 71% than in malignant ones 64%. The percentages of high risk HPV types were higher than those of low risk types in both study groups. Studies concerning this association were conducted using different molecular techniques. Using PCR technique Lai and coworkers, 1992 detected HPV DNA sequences in 50%of benign ovarian tumors, 27.2% of malignant tumors, and one out of eight hepatoma samples which were analyzed as a tissue control[(6)] and these results are compatible to our finding. They suggested that the spread of HPV in the upper genital tract may not be uncommon and may explain the presence of HPV DNA in ovarian tissues. However, their observation was not enough to show whether the virus has got a role in primary ovarian carcinoma or it is only due to an ascending infection. Kuscu and his workers found a higher percentage of HPV DNA (37.5%) in malignant ovarian tumors than in benign tumors (28.1%) (15). Giordano et al., 2008(16) didn't find any correlation between ovarian cancers and the presence of HPV infection using PCR. Other studies have shown HPV DNA in ovarian cancer at lower rate than cervical cancer [(17 & 18)]. Others concluded that identification of HPV DNA in ovarian tumors may provide an evidence of a metastatic cervical cancer (19, 20, & 21). High and low risk type of HPV showed significant difference among study groups. The percentages of high risk types in malignant and benign group were higher than low risk types. This finding reflects a possible role of the high risk virus in the carcinogenesis of ovarian tumors in malignant group or may have a precancerous effect in benign group. The viral type is probably an important factor in this association. Although this may need a large case control study to be proved. In an indirect way this could be related to the patient's genetic makeup. certain host genetic factors, such as polymorphisms or variations in specific human leukocyte antigens (HLA) class II have been implicated in the natural history of HPV infection; some increase the risk of HPV persistence by several folds, whereas others are associated with a reduced risk of persistent HPV infections (22). Beside that HPV intratypic variants in different geographical regions may also determine the association with the

risk of ovarian cancer (23). It has been reported that the distribution of HPV variants varied in different geographical areas, suggesting that the virus and the host have coevolved over time (24). In addition to the host and pathogen genetic variation, the difference of the detection methods employed in the studies might also account for data discrepancy. Studies concerning HPV association with cancer, especially cervical cancers speak about increase viral load. So that, detection of high viral load using PCR for instance is significantly associated with cervical carcinoma (25 & 26). In an infected cell, viral replication ending with release of the virus from cell is definitely against its transformation into a malignant one (27). During viral DNA integration, only E6 and E7 genes remain in the host genome (28). Therefore, their presence in tumor tissues may better represent the real HPV participation in carcinogenesis. For this reason, a hypothesis was made in the present study that if ISH signals for HPV DNA was detected, it ought to be of low intensity so that a high intensity reflects viral replication inside the cell and is against viral participation in ovarian cancer. Opposite to Giordano et al., 2008 who considered that the weak positivity of HPV DNA on PCR analysis is not responsible for ovarian neoplasms (16). In the present study, high percentages were found in the low score categories of HPV16&18. This may reflect a low reproduction (replication) rate of the virus in ovarian epithelial cells. The low intensity of HPV18 DNA signals may also reflect viral latency or past infection with probable viral genome insertion into cellular DNA (16). At the same time, the association of ovarian tumors with HPV16 was more than HPV18**.** Yang in 2003 detected HPV16 in 23.1% and HPV18 in 1.8%of ovarian cancers using real time PCR. He also showed the predominance of HPV16 in ovarian cancers (29). While Low risk HPV DNAs were detected in higher score and intensity in benign tumors significantly differed from that with malignant tumors, which also revealed low score and intensity. HPV6 has been associated with mainly condylomata accuminata and is not generally considered to have a malignant potential (31). Kaufman in 1987 detected HPV6 DNA in 83% of epithelial ovarian tumors by ISH technique, however none of HPV types 16, 18, and 11 DNA could be found in those tissues (5). Lai and colleagues in 1992 (6) also detected HPV6 in ovarian cancers using PCR and Southern blot. While Leake et al. in 1991 did not show HPV types 6 and 11 in archived epithelial ovarian carcinoma tissues using PCR(7). Beckman and colleagues in 1991 failed to reveal HPV DNA in malignant ovarian tumors using PCR with L1 consensus primers (3). McLellan et al., 1990 also failed to detect HPV6, 11, 16, and 18 in ovarian tumors of low malignant potential using primers to amplify E6–E7 region, the sites which are known to be integrated in the host genome (8). Trottier et al., 1995 had found similar results (30); their explanation for the absence of HPV in ovarian carcinoma was that the lack of keratinocytes that express the α6β4 integrin protein which is putatively identified as the receptor for HPV is responsible for their negative results (31). The study results showed that HPV infection may play a relative role in the pathogenesis of ovarian carcinomas or it could facilitate its progression. We should consider data related to HPV type-matching to be an ancillary rather than a definitive data. Our findings warrant future studies to confirm the role of HPV in ovarian carcinoma and the interaction with tumor suppressor genes or other cellular proteins which must be studied with large sample size in Iraqi population. The researchers theorized that the multiple viral infections could act synergistically to increase the pre-malignant mutations or to expedite their progression to cancer. They also suggest that the presence of HPV co-infections may indicate that a woman's immune system is not especially effective in clearing viruses. This new research is the first retrospective study using multiple measurements longitudinally to investigate the relationship between multiple HPV types and carcinogenesis of the ovary. Multiple HPV confections have been reported in the literature. Most of these are double co infections, but triple, quadruple and even quintuple HPV co-infection have also been detected (32, 33, & 34). The results of this study revealed that the percentage of low risk HPV6&11 in benign group was high than malignant group. Significant difference among study groups was detected only in combination of low and high risk HPV types. Only significant difference was found in the presence of combination of both low and high risk HPV types among study groups which may be due to synergistic effect of multiple viral infections. A further study is required to explain the correlation between different HPV types with large sample size.

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