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DNA METHYLATION PATTERN OF L1 GENE OF HIGH RISK HUMAN PAPILLOMAVIRUSES AND IFN-GAMMA AS A CANDIDATE BIOMARKERS FOR PREDISPOSE TO CERVICAL NEOPLASIA IN WOMEN FROM BAGHDAD

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ABSTRACT

Background: Human papillomaviruses (HPV) are the etiological agents of cervical malignancies**.** DNA Methylation of late genes (L1) of high risk human papilloma viruses genotypes (HR- HPVs) takes place regularly in cervical cells and its elevated in high grade lesions and cancer cases when compared to asymptomatic infection or low-grade lesions. Interferons gama(IFN-γ) are antiviral molecules that induce a state of resistance to viral replication in infected and uninfected neighbor cells and regulate the cellular immune response. **Methods**: Fresh cervical tissue biopsy samples were collected from hysterectomy done for women. Extracted DNA from these samples were subjected to amplification using PCR to estimate DNA integrity, viral DNA identification, genotyping of HR-HPVs, sodium bisulfite modification and study of DNA methylation pattern of L1 gene and IFN-γ gene. **Results**: From totally collected 150 samples,90 samples were subjected to all study tests. From them, 24/90 were included in the study of DNA methylation pattern of viral L1 gene; 14/90 samples as control group and 10/90 samples as patient groups whom positive for HPV-16,18 and/or 45. Positivity for DNA methylation of L1 gene was 100% in samples positive to HPV-18 and HPV-45, while it was 66.7%**.** in samples positive to HPV-16.DNA methylation state of IFN-gamma was detected in patients group as 10/10(100%), while the positivity of the DNA methylation of IFN-gamma in the control group was 4/14(28.6%). **Conclusion:** Methylation of L1 gene and IFN-γ gene has a role as a candidate biomarker for the progression of cervical disease.

KEYWORDS: *Human Papilloma Virus, DNA methylation, L1 gene, IFN-gamma, risk factor.*

INTRODUCTION

Cervical carcinoma arises as a consequence of persistent infection with HR-HPV genotypes. Papillomaviruses are small non-enveloped double-stranded DNA viruses with tissue tropism and species specificity[1,2]. The most prevalent mucosal HR-HPV types are HPV-16,HPV-18 and HPV-45 which are responsible of most cervical cancer cases globally[3]. Changes in DNA methylation are central to most cancers; cause defective gene expression, genetic instability, and silencing of mobile DNAs such as jumping genes and viruses. Attempted silencing of mobile DNA can stimulate molecular evolution as variants of the targeted DNA escape repression, which lead to instability of host genetic[4,5].

The realization that viral infections, by inclusion of viral genes into host genomes, can trigger host defense components such DNA methylation machinery initiation has aroused interest for the investigation of epigenetic events happening in both infection and host genomes[6]. It is additionally established that some viruses can discover approaches to adjust distinctive strategies to regulate expression of their genes through modulation of DNA methylation; thus, a virus may silence activation of its genes in a way that favors establishment of persistent infection[7]. Methylation of HPV-DNA considers as a novel biomarker that help to differentiate between benign HPV infections and those that progress to pre-cancer[8]. Detection of epigenetic alteration, especially if related to development of cervical pre-cancer, may consider as a predictive or diagnostic biomarker for risk of cervical cancer among HPV-positive women[9].

Since HPV L1 forms the whole exterior surface of the mature virion, it clearly must mediate initial attachment to host cells. After attachment to cells, L1 must again become flexible enough to finally allow release of the viral genome into a new target cell[10,11].

IFN-gamma has an antiviral, immune-regulatory and anti-tumor characteristics[12,13]. It change transcription in up to 30 genes producing a diversity of physiological and cellular responses. Inhibition of the expression of IFN-gamma results in suppression of cell-mediated local immune defense and stimulates persistent HR-HPV infection of the uterine cervix, which may allow HPV invasion and tumor development[14]. DNA methylation state induced silencing of IFN-γ gene and plays a critical role in the pathogenesis of cervical cancer[15,16].

Current study aimed to evaluate the diagnostic role of DNA methylation pattern of HPV L1gene of HR-HPV in the nature and course of these genotypes in mediating cervical lesions with respect to methylation pattern. Also, to estimate the pattern of IFN-γ methylation in HR-HPV different infections.

MATERIALS AND METHODS

The current cross-section and prospective study was included 150 patients women, fresh cervical tissue biopsy samples(two biopsies per patient) collected from hysterectomy done for those women whom attend to the department of Gynecology of Al-Emamain Medical City at Baghdad, Iraq from March 2016 to March 2017. One biopsy were preserved in the normal saline for DNA extraction and the second one was sent to histopathological lab.

All cervical tissue biopsy samples were diagnosed histo-pathologically. Patients were classified according to the histo-pathological findings into two groups;130 patients with cervical abnormalities group such as (cervical dysplasia (CIN I, II, III), squamous cell carcinoma(SCC), atypical squamous cell and chronic cervicitis) and 20 patients with unremarkable histo-pathological changes whom conceded as negative control group and the hysterectomy done for them for reasons other than cervical abnormalities. Data was taken from each patient include (name, age, smoking state, medical history of patient, family history, marital status and using birth control pills). This study was approved by the ethical committee of the College of Medicine-Al-Nahrain University, Baghdad, Iraq.

DNA extraction. Extraction of Genomic DNA from the cervical tissue biopsy was done using QIAamp® DNA Mini Kit (catalog number 51304, USA). Concentration and purity of DNA of each sample was measured using Nanodrope instrument.

DNA quality estimation using PCR amplification of glyceraldehyde phosphate dehydrogenase(GAPDH). PCR amplification of GAPDH was performed according to Rameshkumar et al[17]. After cycling, the PCR amplified products were electrophoresed in 2% agarose gel. Presence of 240 bp band means positive result (the quality of DNA is good). From the total 150 samples, 90 samples (76 samples of patients and 14 samples of controls) were subject to the farther study tests, while 60 samples were excluded because the quality of DNA is low according to the negative results of testing GAPDH gene.

Detection of the HPV-DNA using PCR. Using PCR amplification with specific primer sets of MY09/MY11[18], detection of HPV-DNA in extracted total DNA was done. After cycling, the PCR amplified products were electrophoresed in 2% agarose gel. The presence of 450 bp band means positive result which indicates the presents of HPV-DNA.

HPV-DNA genotyping. Samples show positivity for the above two reactions were further amplified for genotyping by PCR using specific primer sets for HR-HPV genotypes (16, 18, 31, 35, 45, 51, 52, and 66)[19]. According to previous study[20], results shown that 10/90(11.1%) sample positive for HPV, of them, 5/10 samples positive for HPV-16(chronic non-specific cervicitis, chronic cystic cervicitis, endocervical polyp, CINI and CINII), 1/10 sample positive for co-infection with HPV 16 and 45(Chronic non-specific cervicitis), 2/10 samples positive for co-infection with HPV-16,18 and 45(SCC), 1/10 sample positive for co-infection with HPV-16 and 45(SCC) and 1/10 sample positive for HPV but negative for the genotype sets used in the current study(SCC**)**.

Sodium bisulfite modification. After genotyping, the DNA of 24 positive samples,10/90 samples of patients groups and 14/90 samples of control group, were treated with sodium bisulfite using EpiTect® Bisulfite Kit, catalogue number (59104) according to the manufacturer instruction[21].

Methylation specific PCR (MSP) for detection of DNA methylation pattern of viral L1 gene in HPV-16, 18 and 45. After Sodium bisulfite modification of DNA, methylation pattern of L1 gene of each positive genotype (HPV-16,18 and 45) was detected. Using two primer sets to detect the methylation state of HPV-16[22], two primer sets(one of them for methylated the other for non-methylated state) of HPV-18[23], two primer sets to detect the methylation state of HPV 45[24]. Briefly, PCR master mix was prepared with total volume of 25 μL per one reaction, containing 10 *p*mol forward and reverse primers, 2U of Go Taq ® DNA Polymerase (Promega,USA), 1X of 5X Green Go Taq ® reaction buffer Polymerase (Promega,USA) and 200μM of dNTPs Polymerase (Promega, USA), then nuclease-free water was added until the volume reach to 23μL. Modified DNA (2μL) was added to PCR mixture. No template control (NTC) tube was prepared for each reaction which contains all PCR master mix component but instead of DNA, 2μL of nuclease-free water was added. Amplification was done using PCR thermal cycler (Eppendrowf , Germany). The amplified products were electrophoresed in 2% agarose gel. Presence of band with 150 bp using

methylated primer means presence of methylated state, while the presence of band with 150 bp by using the non-methylated primer means there was no methylation.

Methylation specific PCR for detection of DNA methylation pattern of IFN- gamma gene. Methylation pattern of IFN-gamma gene was detected in all DNA samples (90/90)included in the present study using MSP. Two primer sets, one for methylated state and the other for non-methylated state, of IFN- gamma were used[16]. The amplified products were electrophoresed in 2% agarose gel. Presence of band with 154 bp means presence of methylated state, while the presence of band with 156 bp by using the primer for detection of non-methylation of IFN-gamma gene means there was no methylation.

Statistical analysis. Data were collected, analyzed and presented using two statistical software programs: statistical package for social sciences (SPSS version 22) and Microsoft Office Excel 2013. Numeric variables were presented as mean, standard deviation and range, whereas categorical variables were presented as number and percentage. One proportion Z-test was used to study differences in ratios, Fischer exact test was used to study association between categorical variables, Kruskal Wallis test was used to study differences in mean rank among more than two groups, Odds ratio test was used to estimate risk together with etiologic fraction. Sensitivity, specificity, positive predictive value and negative predictive values were estimated according to equations outlined in Daniel, 2009[25]. P-value was considered significant at ≤ 0.05 and highly significant at ≤ 0.01.

RESULTS

The results of histopathological examination of 24/90 DNA samples included in the study of DNA methylation pattern of viral L1 gene; control group (14/90 samples) and patient groups 10/90 samples; shown that the most common histopathological abnormality was SCC which detected in 4/24(16.7%) patients, (Table 1). The age distribution of included patients ranged from 29 to 72 years with a total mean of 51.17 with SD 12.14.There were no significant statistical differences between different groups of the study according to age (Table 2). Regarding the married status,23/24(95.8%) patients included in detection of DNA methylation pattern of viral L1 gene were married(Table 3). The patients group with highest positivity of family history was SCC group with (4/4 patients,100%) (Table 4).

Regarding the DNA methylation pattern of viral L1 gene that detected in samples positive to HPV-16,18,45 (Table 5),positivity was 100% in HPV-18 and HPV-45, while in the HPV-16

genotype, DNA methylated state was detected in 66.7% (Fig.1). Risk estimation for SCC with respect to genotype was shown in Table 6. The sensitivity, specificity, positive predictive value and negative predictive value of DNA methylation pattern of viral L1 gene for detection of pathology versus control were 100%,71.43%,71.43% and 100%, respectively (Table 7).

DNA methylation state of IFN-gamma was detected in patients group as 10/10(100%),(Fig.1), while the positivity of the DNA methylation of IFN-gamma in the control group was 4/14(28.6%).

Table 1. Classification of patients according to histopathological findings.

Groups according to histopathology		**Patient no.**	**(%)**
Control	Negative	14	58.3
Non-malignant changes	Chronic cystic cervicitis	3	12.5
	Endocervical polyp	1	4.2
Cervical intraepithelial neoplasia	CIN I	1	4.2
	CIN II	1	4.2
Malignant neoplasm	SCC	4	16.7
Total		24	100.0

CIN I,II,III: Cervical Intraepithelial Neoplasia type one, two and three.

SCC: Squamous Cell Carcinoma**.**

Table 2. Mean age of patients according to histopathological findings.

Group	**Mean**	**SD**	**Minimum**	**Maximum**
Negative	50.21	13.42	29.00	72.00
Chronic cystic cervicitis	51.33	8.50	43.00	60.00
Endocervical polyp	34.00	---	---	---
CINI	56.00	---	---	---
CINII	66.00	---	---	---
SCC	53.75	10.40	42.00	63.00
Total	51.17	12.14	29.00	72.00

CIN I,II,III: Cervical Intraepithelial Neoplasia type one, two and three.

SCC: Squamous Cell Carcinoma**.** SD: stander division.

P>0.05 "Kruskal Wallis test"

Table 3. Marital status according to histopathological status.

Group	Married		Unmarried		Total	
	Patient No.	(%)	No.	(%)	No.	(%)
Negative	14	100.0	0	0.0	14	100.0
Chronic cystic cervicitis	2*	66.7	1	33.3	3	100.0
Endocervical polyp	1	100.0	0	0.0	1	100.0
CINI	1	100.0	0	0.0	1	100.0
CINII	1	100.0	0	0.0	1	100.0
SCC	4	100.0	0	0.0	4	100.0
Total	23	95.8	1	4.2	24	100.0

P-value was assessed using one proportion Z test; * significant at P≤ 0.05

Table 4. Positive family history to HPV infection.

Group	Family history					
	Positive		Negative		Total	
	No.	(%)	No.	(%)	No.	(%)
Negative	1	7.1	13**	92.9	14	100.0
Chronic cystic cervicitis	1	33.3	2*	66.7	3	100.0
Endocervical polyp	1	100.0	0	0.0	1	100.0
CINI	0	0.0	1	100.0	1	100.0
CINII	1	100.0	0	0.0	1	100.0
SCC	4	100.0	0	0.0	4	100.0
Total	8	33.3	16	66.7	24	100.0

P-value was assessed using one proportion Z test; *significant at P≤ 0.05; **highly significant at P≤0.01.

Table 5. Genotype expression according to histopathological factors.

Groups	16		18		45		X	
	n	%	n	%	n	%	n	%
Negative	0	0.0	0	0.0	0	0.0	0.0	0
Chronic cystic cervicitis	3	100.0	1	33.3	1	33.3	0.0	0
Endocervical polyp	1	100.0	0	0.0	0	0.0	0.0	0
CINI	1	100.0	0	0.0	0	0.0	0.0	0
CINII	1	100.0	0	0.0	0	0.0	0.0	0
SCC	3	75.0	1	25.0	3	75.0	1	25.0
Total	9	37.5	2	8.3	4	16.7	1	4.2

CIN I,II,III: Cervical Intraepithelial Neoplasia type one, two and three.

SCC: Squamous Cell Carcinoma**.**

X: genotype not included in the present study

Table 6. Risk estimation for squamous cell carcinoma with respect to genotype.

Genotype	SCC (study group = 4)	Others (control group= 20)	P*	OR	95 %CI		EF
					Lower	Upper	
16	3 (75.0%)	6 (30.0%)	0.090	7.00	0.60	81.69	0.29
18	1 (25.0%)	1 (5.0%)	0.186	6.33	0.31	130.76	0.42
45	3 (75.0%)	1 (5.0%)	0.001	57.00	2.76	1176.83	0.74

SCC: squamous cell carcinoma;* Fischer exact test; OR: Odds ratio; CI: confidence interval; EF: etiologic fraction.

Table 7. Statistical analysis of DNA methylation pattern of viral L1 gene for detection of pathology versus control.

Methylation	Pathology n = 10	Control n = 14	Total n =24
Positive	10 (TP)	4 (FP)	14
Negative	0 (FN)	10 (TN)	10
Total	10	14	24

TP: true positive; FP: false positive; FN: false negative; TN: true negative

Sensitivity = TP X 100/ (TP+FN) %= 100%

Specificity = TN X 100/ (FP+TN) %= 71.43%

Positive predictive value (PPV) = TP X 100/ (TP+FP) %= 71.43%

Negative predictive value (NPV) = TN X 100/ (FN+TN) %= 100%

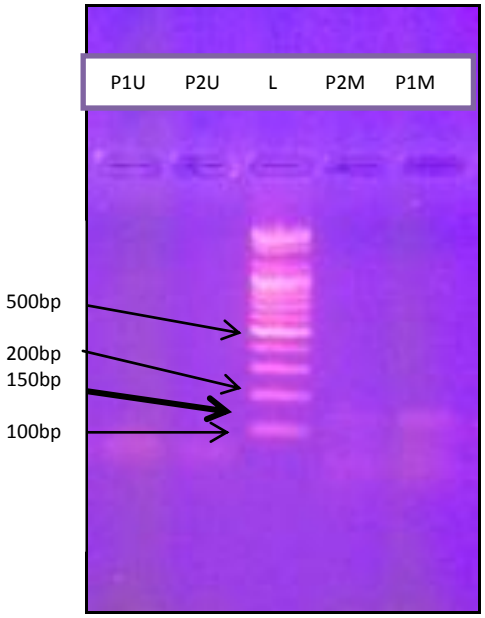

Fig.1. Agarose gel electrophoresis of amplified products of DNA Methylation stat of L1 gene of HPV 18. P1,2:patients, U:unmathylated L1 gene, M: methylated L1gene, L:100bp ladder.

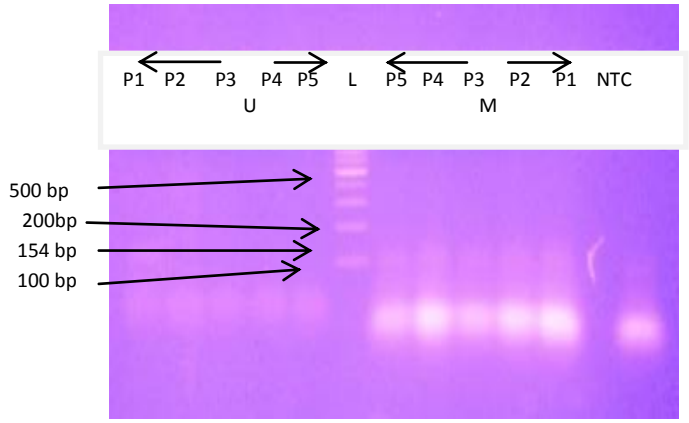

Fig. 2. Agarose gel electrophoresis of amplified products of DNA Methylation stat of IFN-gamma gene. P1,2,3,4 and 5:patients,U:unmathylated gene, M: methylated gene, L: 100bp ladder.

DISCUSSION

Human papillomavirus causes most of the cervical cancer,HPV-16 and HPV-18 are involved in approximately 70 % of the cases leading to cervical carcinoma[26,27]. The importance of detection of viral DNA methylation pattern due to the fact that this pattern could be used as early predictive molecular marker for cervical carcinoma and other cervical abnormalities[28].

In the current study, the mean age of patients was 51.17years and that agreed with other studies done by Hassan et al. (2015) and Shirish et al.(2014)[29,30]. Histopathological findings demonstrated that the most common type of cervical carcinoma was SCC and that agreed with the result reported in a study by Misra et al.(2009) which includes 4176 patients and referred to that the prevalence of the carcinoma of the cervix was 4.8%, while the prevalence of intraepithelial lesion was 2.6% in the same group[31]. In the second study by Kalyani *et al* (2010), the total number of samples was 19615, the total malignant results was 2744 case,1544 case of them were in female group which represent 56.3%, the SCC percentage was 262/271case of the cervical cancer and this was the highest group of cervical abnormalities[32].

In the current study, L1 gene was methylated in 6/9(66.7%) patients positive for HPV16, from them, DNA methylated state was present in only 1/3(33.33%) patient related to histopathological group SCC positive for HPV16, and that differed from other studies[33-37]. This may be due to difference in the sample size and geographical distribution.

Kalantari et al.(2014) were studied CpG methylation of the L1 open reading frame; they found increased methylation in cervical carcinomas, while methylation was relatively decreased in low grade cervical intraepithelial neoplasia and normal samples[24]. This finding was confirmed in two follow-up studies, one using laser capture micro dissection to evaluate three cancer samples[33] and the other study of DNA methylation of L1 region and chromosomal integration in a small set of abnormal Pap smear samples[34]. Fernandez et al.(2009) were detect increase methylation state in L1 and L2 regions with tumorigenesis in samples collected from 18 women[35]. These results are consistent with the CpG methylation patterns that observed in the late genes in a study in Costa Rican population, a case- control study within a large prospective cohort. Diagnostic specimens were collected at the time of CIN III, persistent HPV16 infection, pre-diagnostic specimens at the first HPV16-positive screening visit, and control specimens from women with infection clearance within 2 years, DNA methylation levels at 67 CpG sites throughout the HPV16 genome was studied using pyrosequencing[36], these studies indicated that the methylation increased with progression of the disease.

Methylation state of the L1 region in patients positive to HPV18 was 100% in the cervical abnormalities cases (2/2), one of those patients had SCC. One of the first studies on HPV18 was by Badal et al.(2004), whom used bisulfite sequencing of cloned molecules to demonstrate the methylation pattern of the L1 region in cervical carcinoma samples. They found hyper-methylation in L1 amongst both cancers and normal samples[37]. Turan et al.(2006) studied HPV18 DNA methylation in L1 gene and URR; they found low levels of methylation distributed throughout the URR in all samples, methylation levels were low in normal samples, with only sporadic methylation observed in the L1 region, while two high-grade lesions and most cancer samples showed hyper-methylation in the L1 region[38], and this result was in agreement with the result of the present study. A report by Fernandez et al.(2004) analyzed all CpG sites in the HPV18 genome using cell lines and bisulfite sequencing of cloned fragments from samples without neoplasia and some primary cervical carcinoma samples, they found increased methylation of HPV18 in L1 region in carcinoma cases when compared with specimens with no detectable abnormality[35], and this result was in agreement with the result of the present study.

In the current study, HPV L1 gene methylation pattern of 4/24 patients positive for HPV-45 was 100%, one of those patients had chronic non-specific cervicitis, the other three patients

had SCC. The results of hyper-methylation in the cervical carcinoma in the present study was in agreement with results of Wentzensen et al.(2012)[39]. Also the data of the present study was in contrast to the results of the Mina et al.(2014) whom referred to that there were 12 patients positive for HPV-45, six of those patients had invasive cancer, all cancer cases show methylation in 102/108 CpG of L2/L1 which represent (94.4%)[40].

The role of IFN gamma has been studied extensively and there was evidence that intra-lesional IFN gamma might be a prognostic marker for clearance of HR-HPVs[41].

In the current study, DNA methylation of IFN gamma gene was detected in 10/10 (100%) patients whom were positive for HPV16,18 and 45, while it was detected in 4/14 (28.6%) patients of control group and that in agreement with result of study by Dong et al.(2014)[42], whom suggested that that most cervical cancer samples exhibited hyper-methylation of IFN gamma gene in contrast to control samples and this indicate that cervical tumorigenesis may be associated with DNA hyper-methylation in the promoter region of the IFN gamma gene.

CONCLUSIONS

Methylation of L1 gene and IFN-γ gene has a role as a candidate biomarker for the progression of cervical disease.

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