RESEARCH COMMUNICATION

Elevation of HPV-18 and HPV-16 DNA in the Plasma of Patients with Advanced Cervical Cancer

Mansooreh Jaberipour[1], Alamtaj Samsami[2], Farahnaz Sahraiian[2], Talieh Kazerooni[2], Masoomeh Hashemi[2], Abbas Ghaderi[1,3], Mojtaba Habibagahi[3*]

Abstract

 Objective: Cervical cancer (CC) is one of the main problems in women's health in which the pathologic role of the human papilloma virus, HPV, is undeniable. Molecular methods have shown viral DNA in affected tissues, related to the disease progression. **Patients and Methods**: We here studied 100 patients with abnormal Pap test results. HPV DNA loads in the plasma samples were measured by quantitative real time PCR, using specific primers and probes for the E6 genes of HPV types 16, 18, 33 and 52. Another 50 women with no obvious malignancy were enrolled as controls. **Results**: Pathological studies revealed 81 patients with CC and 19 with cervical intraepithelial neoplasia. Only 19 of the cancer patients (15 with squamous cell carcinomas and 4 with adenocarcinomas) had detectable genetic material of HPV-16 (N=4) and HPV-18 (N=15) in their plasma; genetic material of other types was absent. HPV DNA copies increased with advanced disease in both types. Significantly smaller amounts of HPV DNA of types 16 and/or 18 were detected in the plasma of 16% of the controls while other types were negative. **Conclusion**: The evidence of HPV DNA of high risk types in the plasma of women with CCs strongly emphasizes the necessity of more longitudinal comprehensive studies to determine its role as a possible biomarker in cervical cancer.

Keywords: HPV-16 DNA- HPV-18 DNA - plasma - cervical cancer cases - Iran

Introduction

 Every year, more than half a million women develop cervical cancer, half of whom may die eventually (Franceschi, 2005). Several studies have shown the major etiologic role of the human papilloma virus (HPV) infection in neoplasia of cutaneous and mucosal epithelia (Castle et al., 2005; Khan et al., 2005; Kjaer et al., 2006). HPV infection is a common sexually transmitted contamination and both sexes can be infected with the virus. Infection usually clears within a few months and about 90% of the cases became clear within two years of infection (Broomall et al., 2010). However, a small proportion of infections may persist and can progress to cancer in women (Bosch and de, 2002). Of more than 200 types of HPV identified so far, about 35 have shown to be associated with ano-genital neoplasia disorders. Based on such evidence, some HPV types (such as 6, 11, 42, 43 and 44) could be categorized as low risk types, responsible for condyloma acuminate and laryngeal papilloma while some others such as HPV-16 and HPV-18 are considered as high risk viruses, associated with cancers of ano-genitalia and cervix (Smith et al., 2007). In accordance with such partitioning, evidence shows the expression of

the two HPV gene products, E6 and E7, in cervical cancers which can inactivate the host p53 and Rb, respectively, and therefore could cause cancer in the infected women (Sima et al., 2008).

 In this regard, almost all cases of cervical cancer and more than 90% of condyloma acuminate bear HPV DNA in their affected tissues. This explains why detection of viral DNA has become one of the key practical screening and follow-up methods (Franceschi and Clifford, 2005). Ordinary PCR-based techniques using consensus primers (MY09 and MY11 amplifying L1 sequence of HPV) readily allow detection of HPV DNA as low as 1000 to 10000 copies of viral genome in tissue samples (Chaiwongkot et al., 2007). Restriction fragment length polymorphism (RFLP) analysis of such amplicons could identify the type of HPV and modifications to these techniques (e.g. nested PCR) have enhanced their ability and broadened their detection spectrum (Nobre et al., 2008). In addition to the affected tissues, the presence of viral nucleic acid sequences has been revealed in the peripheral blood and sera of advanced cervical cancer patients (Wei et al., 2007). Similar studies have proposed the HPV viral load as a determining factor for progression of cervical cancer (Dong et al., 2002; Ho et al., 2005;

*[1]Shiraz Institute for Cancer Research, [2]Department of Gynecology and Obstetrics, [3]Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran *For correspondence : agahim@sums.ac.ir, habibagahim@yahoo.com*

Tsai et al., 2005). These studies have suggested that the circulating HPV can provide an early biomarker to identify the patients at the risk for developing metastases. Accordingly, several groups have tried to quantify HPV-16 and HPV-18 viral loads in the plasma of cervical cancer patients of different stages (Dong et al., 2002; Ho et al., 2005; Tsai et al., 2005; Wei et al., 2007). Similarly, the high prevalence of HPV-52 in the blood sample of cervical cancer patients has been reported, showing an increase in the viral burden as cancer stage develops from cervical intraepithelial neoplasias (CIN) to invasive cancer (Ho et al., 2005). Therefore, in this study we investigated the viral load of some high risk types of HPV (16, 18, 33 and 52) in the plasma samples of women with CIN, cervical cancer and a group of women without evident signs of malignancy as control in southern of Iran and correlated that with the patients' clinical data.

Materials and Methods

Patients and samples

From July 2008 to July 2009, hundred eligible patients with confirmed histopathological diagnoses of different stages of cervical malignancy and cervical intraepithelial neoplasia (CIN) at the Gynecology and Oncology Clinics of the Shiraz University of Medical Sciences were recruited. The most common causes or symptoms of their referral to the gynecology clinics were post-coital or post-menopausal pain, hypermenorrea, discharge, dyspareunia, abnormal uterine bleeding and urinary obstruction. All the patients underwent complete physical and gynecologic examinations and their disease was staged according to the guidelines of the International Federation of Gynecology and Obstetrics (FIGO).

Preparation of plasma samples and DNA extraction

EDTA-anticoagulant blood samples were collected at the time of the interview by venipuncture from the patients and controls. The samples were centrifuged at 750Xg for 10 min, and the plasma fractions were stored at -20°C. DNA from plasma samples was extracted, using the QIAmp Ultra Sense Virus Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany).

Quantitative real-time PCR

Commercial kits for quantification of human Papilloma Virus genomes (Advanced kit version, PrimerDesign, Southampton, UK), with specific primers and probes for E6 gene of HPV-16, -18, -33 and -52, were used according to the manufacturer's protocol. The kits were designed to detect 6-12 strains of each HPV type. Real-time PCR reactions were set up in a reaction volume of 20 μl, using the TaqMan Universal PCR Master Mix (ABI, Perkin Elmer, USA). DNA amplifications were carried out in a Chromo4 Real-time PCR Detector instrument (Bio-Rad, Foster city, CA, USA). Amplification reactions were done in triplicates for HPV-16, -18, -33 and -52 separately, and each experiment had its own standard curve. Standard curves were also run in parallel with every analysis using positive control template, according to the manufacturer's guide. Concentrations of the circulating

HPV DNA were expressed as copies of HPV genome/ml of plasma and were calculated from the regression equation using the individual Ct (threshold cycle number) values. Quantification of HPV in the plasma of the controls that were plasma HPV+ was repeated after two months.

Statistical analysis

The calculated results are shown as medians of the three replicates. P-values less than 0.05 were considered as statistically significant in all the analyses. Differences in values were evaluated using Mann–Whitney U test and student t-test. Statistical analysis was performed using the Statistical Package for the Social Sciences Software version 11.5 (SPSS, Chicago, IL, USA). Graphical presentation was plotted and analyzed, using Prism 4 software (Graphpad Inc; San Diego CA, USA).

Results

Patients and treatment modalities

In this study we recruited hundred eligible patients. Of 19 patients diagnosed with CIN, 10 were with CIN I, one was with CIN II, and eight were with CIN III. Pathological studies on patients with cervical cancer confirmed the diagnosis of squamous cell carcinoma in 69 patients (stage I=12, stage II=28, stage III=20, stage IV=9) and adenocarcinoma in the other 12 patients (stage I=2, stage II=4 and stage III=6). All the patients underwent primary radiotherapy and/or surgery, with or without concurrent chemotherapy. Apart from four patients with CIN, blood samples were taken retrospectively after assigned treatments. Treatment protocols were based on four modalities of surgery, surgery and radiotherapy, chemotherapy and both chemotherapy and radiotherapy after the surgery. Fifty women, referred to the gynecology department for routine checks, without apparent pathological findings of CIN or cancer of the cervix, were also registered as control and underwent physical and gynecologic examinations. The mean age of the women in the two groups of patients and control was 46.5 years (ranging between 29 to 77) and 41 years (ranging between 23 to 77), respectively. The median parity of the studied patients was 5 (ranging between 0-13), and that of the control group was 3 (ranged between 0-11). This study was reviewed and approved by the ethics committee of the University and informed consent was obtained from all the participants at the time of the recruitment.

A combination of surgery, radiotherapy and chemotherapy was applied for treatment protocol. Of 100 registered patients, 16 received only radiotherapy while treatment of the 10 other patients was based on a combination of chemotherapy and radiotherapy but not surgery. Treatment of some patients after surgery was followed by radiotherapy (34%) or combination of radiotherapy and chemotherapy (14%) while the rest (22%) did not continue these therapeutic regimens. Four patients with CIN I did not accept any therapies after their diagnosis.

Patients with Cervical cancer diagnosis

Based on pathological studies, cervical cancer of

Table 1. List of Plasma HPV+ Patients and Controls

Age	HPV type	Histological diagnosis	Stage	Metastases	Adjuvant therapy
49	16	SCC	IIB	+	S+R
70	16	SCC	IVB	+	Ch+R
49	16	SCC	IIB	+	S+R
55	16	AC	IIB	+	S
46	18	AC	IIIB	+	S+R+Ch
65	18	SCC	IIB	+	S+R
49	18	SCC	IB	-	S
53	18	AC	IIB	+	S+R
70	18	SCC	IVB	+	Ch+R
46	18	SCC	IB	-	S
49	18	SCC	IIB	+	R
49	18	AC	IIIB	+	S+R+Ch
56	18	SCC	IB	-	S+R
38	18	SCC	IIIB	+	S+R
46	18	SCC	IIB	+	S+R
54	18	SCC	IB	-	S
46	18	SCC	IIB	+	R
58	18	SCC	IIIB	+	R
43	18	SCC	IVB	+	R
55	18	C	-	-	-
41	18	C	-	-	-
45	18	C	-	-	-
52	18	C	-	-	-
30	18	C	-	-	-
40	16	C	-	-	-
43	16+18	C	-	-	-
31	16+18	C	-	-	-

AC, adenocarcinoma; CC, cervical cancer; SCC, squamous cell carcinoma; S, surgery; R, radiotherapy; Ch, chemotherapy; C, control
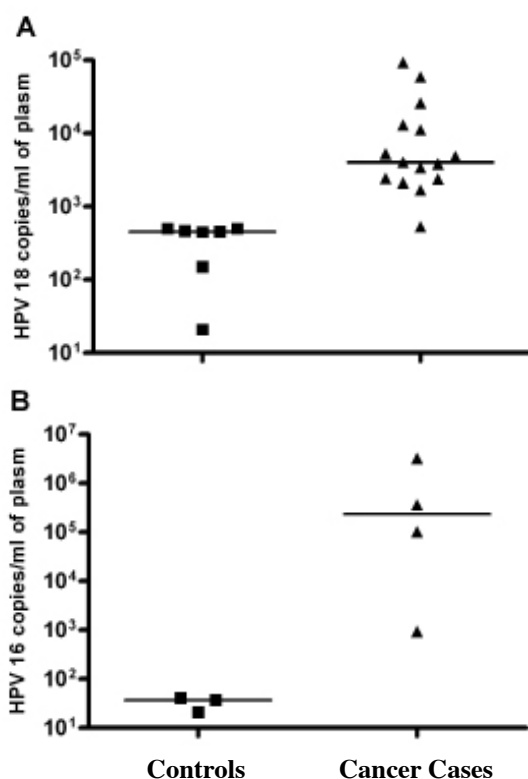

Figure 1. Comparison of the HPV 18 (A) and HPV 16 (B) DNA Copy Numbers in Plasma from Women with Cervical Cancer and Controls. Number of copies of HPV DNA molecules in the plasma were determined by quantitative real-time PCR using specific primers and probes to E6 gene of each type

different stages was diagnosed in 81 patients. A significant correlation was found between the age of the patients and the stage of their cervical cancer where older patients showed higher grades of the disease (P=0.0008, r=0.7188). A significant percentage of the patients with cervical cancer (67%) had started their sexual activity before age 16 (p=0.021). Moreover, almost 80% of the patients had a history of giving birth to 3 or more babies at the time of the study (p=0.005). A negative significant correlation was also found between the patients' level of education and the presence of cervical cancer (P=0.0006). Other findings such as age at menarche, infertility and smoking habit were not associated with cervical cancer.

The Presence of HPV DNA in the Plasma

Commercial quantitative real time PCR kits with Primers and probes specific to HPV E6 gene of types 16, 18, 33 and 52 were used to detect viral HPV DNA in the plasma samples. Table 1 lists the corresponding data regarding 19 (23.5%) out of 81 patients that burdened HPV genetic materials in their plasma. Only genetic materials of HPV-16 (in 4 cases, 5%) and HPV-18 (in 15 cases, 18.5%) were found in the plasma of the patients, while genetic materials of other studied types were not detectable. That is, almost 79% of plasma HPV+ patients had HPV-18 DNA in their serum. The majority of the plasma HPV+ patients (79%) were diagnosed as squamous cell carcinoma. Among the patients, the median DNA copy numbers of HPV-16 and HPV-18 in each milliliter of the plasma were 234395 and 4040, respectively. The plasma viral loads of both HPV DNA types showed a significant increase with the advanced disease stages (P=0.004).

Neither of the patients diagnosed as CIN had detectable amounts of HPV DNA of any tested types in their plasma sample; however, we could show different amounts of viral DNA of types 16 and/or 18 in the plasma samples of 8 (16%) women in the control group. Five of these women had HPV-18 (10%), 1 showing the evidence of HPV-16 (2%) and 2 cases being positive for both HPV-16 and -18 (4%). That is, at least 87.5% of the plasma HPV+ women in this group had traces of HPV-18 DNA in their blood. As Figure 1A shows, the median concentration of HPV-18 DNA (450 copies/ml of plasma) in women in the control group was significantly lower (P = 0.0021) than the studied patients (4040 copies/ml). Similarly, as depicted in Figure 1B, fewer HPV-16 DNA molecules were found in the plasma of the controls than in the patients (37 copies/ml vs. 234400 copies/ml of plasma). Re-quantification of the plasma HPV after two months, in those controls that showed to be plasma HPV+, demonstrated fluctuations in their viral loads; however, they were never become virus clear during the study.

Moreover, no significant correlation was found between age, education, sexual activity, number of pregnancies, age at menarche, infertility and smoking with HPV-16 or HPV-18 DNA load.

Discussion

The results of this study showed an exclusive presence of DNA from HPV-16 and HPV-18 in the plasma of 23.5%

of the patients with histopathological diagnosis of cervical cancer while some other tested types such as HPV-33 and HPV-52 were absent. Moreover, the same HPV types were also detected in the plasma samples of some control women (16%) with no sign of malignancy, albeit in lower quantities. The presence of different types of HPV in the peripheral blood mononuclear cells (PBMCs), sera and plasma of cancer patients, as well as cancerous tissue, has been indicated by different investigators (Dong et al., 2002; Ho et al., 2005; Tsai et al., 2005; Wei et al., 2007). Previous studies have suggested that the circulating HPV DNA in the peripheral blood of the cervical cancer patients might arise from the lysis of the circulating cancer cells or micrometastases shed by the tumor (Dong et al., 2002; Ho et al., 2005; Tsai et al., 2005; Wei et al., 2007). However, the presence of HPV DNA in 8.3% of the healthy blood donating men (Chen et al., 2009) or detection of plasma viremia in significant percentages of apparently healthy women (Yang et al., 2004) could show the existence of other mechanisms as well. We also found HPV DNA of types 16 and 18, but not other high risk tested types, in the plasma of 16% of women with no history of malignancy at the time of examination. Although these women had no symptoms of cervical cancer, some had minor signs of inflammation or bacterial infection in their pap-smear test. It would be possible that such conditions facilitate the spread of the existing virus into the blood circulation even in the absence of malignancies. In fact, Ma et al. (Ma et al., 2009) have elegantly demonstrated the presence of the HPV-16 viral genome and the whole viral particles inside the bacteria recovered from the cervical cancer biopsies. Based on such findings, they have suggested that bacterial infection can form a source for persistence of HPV virus which can play a major role in cervical malignancy as well. This may also show the risk of occurrence of future malignancy in the plasma HPV+ women if other conditions are provided.

A diverse rate of HPV detection in the plasma of patients with cervical cancer has been reported in the literature (Dong et al., 2002; Ho et al., 2005; Tsai et al., 2005; Wei et al., 2007; Yang et al., 2004). In many of the earlier reports, PCR or nested PCR have been used to detect HPV genes. Using HPV consensus primers, MY09/11, Sathish et al. (Sathish et al., 2004) detected HPV in 11.8% of the patients while nested PCR on the E6 or L1 genes increased the rate of detection to more than 24% and 65%, of the patients, respectively (Kay et al., 2005). Application of real-time PCR has made this measurement more quantitative and accurate where Gnanamony et al. detected HPV-16 and HPV-18 in 54% of the patients (Gnanamony et al., 2010). We also used similar method with probes and primers; however, the rate of detection was half of that was found in India. In our study, the HPV viral DNA was not detected in the plasma of any patient with CIN diagnosis at any stage and even after the surgery. Similarly, in a study by Ho et al., circulating HPV DNA was not present in the plasma samples from the patients with CIN lesions (Ho et al., 2005). On the contrary, a report from Tsai et al. showed the presence of HPV-16 and HPV-18 DNA in the peripheral blood of CIN patients; however, in quantities relatively

less than cervical cancer patients (Tsai et al., 2005). Such discrepancy can be explained by fluctuation of HPV DNA loads in the plasma, as we also observed in some women in the control group. Furthermore, finding HPV DNA in the plasma, mainly from the high risk types, could be an important result as it can suggest the possibility that those women may be at a high risk of developing severe disease and cancer. Larger groups of CIN patients of different stages in longitudinal studies should be examined to resolve this uncertainty.

Several groups have studied the HPV types in tissues from cervical cancer patients in Iran (Mortazavi et al., 2002; Esmaeili et al., 2008). In a report by Mortazavi et al. (Mortazavi et al., 2002), the prevalence of different types of HPV from the cervical lesions of the patients in the capital of Iran was similar to those reported in other regions of the world. Accordingly, HPV-16 was the most common type associated with cervical cancer followed by HPV-18 and HPV-33. In northern Iran, HPV-16 is still the most prevalent type in the cervical lesions; HPV-18, and HPV-33 are also frequent types (Esmaeili et al., 2008). However, there is no report of finding HPV-52 among Iranian patients with cervical cancer. Farjadian S. et al. worked on the tissue samples from patients with cervical carcinoma of the same geographical region of our study (southern of Iran); only 26% of the tissue samples had HPV-16 but not HPV-18 (Farjadian et al., 2003). Accordingly, they concluded that HPV-16 and HPV-18 were not the most frequent types of HPV among the patients with cervical carcinoma in the region. In the plasma samples of our patients, however, we found HPV-18 DNA (18.5%) and less frequently HPV-16 DNA (5%) but not other types that we tested. Unfortunately, DNA samples extracted from the lesions of those patients were not available and we only typed the plasma samples. It is believed that the cervical viral infection is the source of viral DNA in the plasma. In fact, there has been 100% concordance between the sequences obtained from the plasma samples and tissue HPV (Yang et al., 2004; Gnanamony et al., 2010). Therefore, with significant precautions, we may consider HPV-18 as the more frequent type of HPV in our patients in southern Iran. To the best of our knowledge, our report is the first information of quantitative measurement of HPV DNA in the plasma of the Iranian patients although these data are not in full agreement with those of the previous reports from carcinoma tissue samples.

In overall, these data reconfirm the possibility of finding HPV DNA of the high risk types in the plasma of women, even without apparent signs of cervical cancer whereas higher numbers of the viruses were present in the plasma from the patients with cervical cancer malignancy. More longitudinal comprehensive studies with larger scales of participants are required to elucidate if the presence of such viruses in non-cancer women could be considered as a biomarker for a high risk of cervical cancer.

Acknowledgments

References

Bosch FX, de Sanjosé S.(2002). Human papillomavirus in cervical cancer. *Curr Oncol Rep*, **4**, 175-83.

Broomall EM, Reynolds SM, Jacobson RM (2010). Epidemiology, clinical manifestations, and recent advances in vaccination against human papillomavirus. *Postgrad Med*, **122**, 121-9.

Castle PE, Solomon D, Schiffman M, et al (2005). Human papillomavirus type 16 infections and 2-year absolute risk of cervical precancer in women with equivocal or mild cytologic abnormalities. *J Natl Cancer Inst*, **97**, 1066-71.

Chaiwongkot A, Pientong C, Ekalaksananan T, et al (2007). Evaluation of primers and PCR performance on HPV DNA screening in normal and low grade abnormal cervical cells. *Asian Pac J Cancer Prev*, **8**, 279-82.

Chen AC, Keleher A, Kedda MA, et al (2009). Human papillomavirus DNA detected in peripheral blood samples from healthy Australian male blood donors. *J Med Virol*, **81**, 1792-6.

Dong SM, Pai SI, Rha SH, et al (2002). Detection and quantitation of human papillomavirus DNA in the plasma of patients with cervical carcinoma. *Cancer Epidemiol Biomarkers Prev*, **11**, 3-6.

Esmaeili M, Bonyadi M, Dastranj A, et al (2008). HPV typing in women with cervical precancerous and cancerous lesions in northwestern Iran. *Gynecol Obstet Invest*, **66**, 68-72.

Farjadian S, Asadi E, Doroudchi M, et al (2003). High risk HPV types in southern Iranian patients with cervical cancer. *Pathol Oncol Res*, **9**, 121-5.

Franceschi S (2005). The IARC commitment to cancer prevention: the example of papillomavirus and cervical cancer. *Recent Results Cancer Res*, **166**, 277-97.

Franceschi S and Clifford GM (2005). Re: a study of the impact of adding HPV types to cervical cancer screening and triage tests. *J Natl Cancer Inst*, **97**, 938-9.

Gnanamony M, Peedicayil A, Subhashini J, et al (2010). Detection and quantitation of HPV 16 and 18 in plasma of Indian women with cervical cancer. *Gynecol Oncol*, **116**, 447-51.

Ho CM, Yang SS, Chien TY, et al (2005). Detection and quantitation of human papillomavirus type 16, 18 and 52 DNA in the peripheral blood of cervical cancer patients. *Gynecol Oncol*, **99**, 615-21.

Kay P, Allan B, Denny L, et al (2005). Detection of HPV 16 and HPV 18 DNA in the blood of patients with cervical cancer. *J Med Virol*, **75**, 435-9.

Khan MJ, Castle PE, Lorincz AT, et al (2005). The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J Natl Cancer Inst*, **97**, 1072-9.

Kjaer S, Hogdall E, Frederiksen K, et al (2006). The absolute risk of cervical abnormalities in high-risk human papillomavirus-positive, cytologically normal women over a 10-year period. *Cancer Res*, **66**, 10630-6.

Ma Z, Liu L, Zhang F, et al (2009). Human papillomavirus type 16 exists in bacteria isolated from cervical cancer biopsies. *J Int Med Res*, **37**, 1065-74.

Mortazavi S, Zali M, Raoufi M, et al (2002). The prevalence of human papillomavirus in cervical cancer in Iran. *Asian Pac J Cancer Prev*, **3**, 69-72.

Nobre RJ, de Almeida LP, Martins TC (2008). Complete genotyping of mucosal human papillomavirus using a restriction fragment length polymorphism analysis and an original typing algorithm. *J Clin Virol*, **42**, 13-21.

Sathish N, Abraham P, Peedicayil A, et al (2004). HPV DNA in plasma of patients with cervical carcinoma. *J Clin Virol*, **31**, 204-9.

Sima N, Wang W, Kong D, et al (2008). RNA interference against HPV16 E7 oncogene leads to viral E6 and E7 suppression in cervical cancer cells and apoptosis via upregulation of Rb and p53. *Apoptosis*, **13**, 273-81.

Smith JS, Lindsay L, Hoots B, et al (2007). Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer*, **121**, 621-32.

Tsai HJ, Peng YW, Lin LY, et al (2005). An association between human papillomavirus 16/18 deoxyribonucleic acid in peripheral blood with p16 protein expression in neoplastic cervical lesions. *Cancer Detect Prev*, **29**, 537-43.

Wei YC, Chou YS, Chu TY (2007). Detection and typing of minimal human papillomavirus DNA in plasma. *Int J Gynaecol Obstet*, **96**, 112-6.

Yang HJ, Liu VW, Tsang PC, et al (2004). Quantification of human papillomavirus DNA in the plasma of patients with cervical cancer. *Int J Gynecol Cancer*, **14**, 903-10.