Human papillomavirus DNA in plasma of patients with HPV16 DNA-positive uterine cervical cancer

Takako Shimada[1], Naohiro Yamaguchi[2], Noriyuki Nishida[2], Kentaro Yamasaki[1], Kiyonori Miura[1], Shigeru Katamine[3], Hideaki Masuzaki[1]

[1] Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

[2] Department of Molecular Microbiology & Immunology, Nagasaki University Graduate School of Medicine, 1-12-4 Sakamoto, Nagasaki, 852-8523, Japan

[3] Nagasaki University Graduate School, 1-14 Bunkyo, Nagasaki, 852-8521, Japan

Corresponding author:

Takako Shimada, MD,

Department of Obstetrics and Gynecology,

Nagasaki University Graduate School of Biomedical Sciences,

1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Phone: +81-95-819-7363

Fax: +81-95-819-7365

Email: shimachan-ngs@umin.ac.jp

Running Title: human papillomavirus DNA in the plasma

Abstract

Objective : The squamous cell carcinoma antigen (SCCA) is considered the most accurate serologic tumor marker for uterine cervical carcinoma. However, serum SCCA levels were found to correlate significantly with clinical severity of atopic dermatitis and chronic renal failure. The present study was conducted in patients with human papillomavirus (HPV)16 DNA-positive uterine cervical cancer to determine the plasma level of HPV16 DNA and the diagnostic values of plasma HPV DNA in these patients.

Methods : Forty-three HPV16-positive patients with cervical intraepithelial neoplasia (CIN) or uterine cervical SCC were recruited in this study. The diagnosis was cervical cancer in 20 patients, high-grade squamous intraepithelial lesions (HSIL) in 21, low-grade squamous intraepithelial lesions (LSIL) in 1 and negative for intraepithelial lesion or malignancy (NILM) in 3 patients. Before any treatment, blood samples were collected from all patients. For analysis of HPV DNA in plasma of patients with cervical cancer, qPCR fluorescent assay for HPV16 was performed using HPV16 primers and SYBR Green dye using the LightCycler 480 SW1.5 apparatus.

Results : Plasma HPV16 DNA was detected in only 30.0% of patients with HPV16-positive cervical cancer and in none of normal controls. The copy number of plasma HPV16 DNA was higher in patients with invasive cancer than in those with cervical intraepithelial neoplasia (CIN3), microinvasive cancer and in normal individuals.

Conclusions : These results indicated that plasma HPV DNA level could be potentially used as a marker of low-invasive cervical cancer tumors in patients with normal SCCA levels before treatment.

Introduction

The squamous cell carcinoma antigen (SCCA), a SCC tumor-associated protein, was first discovered in uterine cervical SCC by Kato and Torigoe in 1977 (1). SCCA is expressed not only in SCC but also in normal squamous epithelium (2) and considered the most accurate serological marker of uterine cervical carcinoma. However, serum levels of SCCA have been reported also to correlate with the clinical severity of atopic dermatitis, chronic hypertension, and chronic renal failure (3).

Epidemiological studies have provided data on the incidence of human papillomavirus (HPV) infection and the risk factors for HPV infection and genital precancerous lesions (4-6). The long duration of HPV infection is attributed to the ability of the virus to subvert innate immune responses (7, 8). Persistent infection with HPV confers a strong risk for development of subsequent neoplasia (9, 10). Several groups have examined the prevalence of HPV DNA in plasma of cervical cancer patients, although there is discrepancy in the results of these studies (11, 12, 13, 14). The different prevalence rates could be due to the different populations examined, different HPV types, and different methods used for the detection.

The present study was designed to determine the prevalence of HPV16 DNA in plasma of patients with HPV16 DNA-positive uterine cervical cancer and its diagnostic and prognostic value in these patients.

MATERIALS AND METHODS

Patients and sample collection

This study was approved by the Human Ethics Committee of Nagasaki University Hospital. Forty-three HPV16-positive patients with cervical intraepithelial neoplasia (CIN) or uterine cervical SCC were recruited in this study. All patients were examined and/or treated at Nagasaki University Hospital between December 2007 and June 2008. Each patient underwent pelvic examination followed by conventional cervical cytology and determination of serum SCCA level (*see below*). After obtaining informed consent, specimens for HPV typing were harvested (SurePath & CytoRoch, MBL) and evaluated for HPV DNA by polymerase chain reaction.

Table 1 shows the stage of cervical cancer and treatment modalities. The diagnosis was cervical cancer in 20 patients, high-grade squamous intraepithelial lesions (HSIL) in 21, low-grade squamous intraepithelial lesions (LSIL) in 1 and negative for intraepithelial lesion or malignancy (NILM) in 3 patients. The stage of cervical cancer was CCIa1 in 4 patients, CC Ia2 in 1, CC Ib1 in 2, CC Ib2 in 2, CC IIa in 4, and CC IIb in 4, CC IIIb in 1, CC IVa in 1, CC IVb in 1, according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. We also recruited 20 normal individuals who had no history of cervical dysplasia or

neoplasia, as negative controls. Subjects of the control group were confirmed by cervical cytology to be free of cancer or intraepithelial lesions and by hybrid Capture II test to be HPV DNA-negative. Before any treatment, 7-ml blood samples were collected from all patients and placed immediately into tubes containing ethylenediaminetetraacetic acid (EDTA).

SCCA assay

Serum SCCA levels were assayed by radioimmunoassay (RIA, SRL, Tokyo). The normal value of SCCA in our hospital is ≤1.5 ng/ml.

Isolation of DNA

Blood samples were centrifuged at 3,000 rpm for 5 min at room temperature. The plasma was saved and stored at -20ºC until analysis. The DNA was extracted from 200 μl of plasma using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany) and the DNA was eluted with 50 μl of milliQ.

Real-time qPCR analysis of HPV-DNA

For analysis of HPV DNA in plasma of patients with cervical cancer, quantitative real time PCR (qPCR) fluorescent assay for HPV16 was performed using HPV16 primers and SYBR Green dye using the LightCycler 480 SW1.5 apparatus (Roche Molecular Biochemicals). HPV16 primers were as follows. HPV16E6E7 forward primer: 5'-ATC ATC AAG AAC ACG TAG AG-3'; reverse primer: 5'-GAT CAG TTG TCT CTG GTT GCA AAT-3'. (II) HPV16E7-forward primer:

5’CAGCTCAGAGGAGGAGGATG-3’; reverse primer: 5’-ATTGTAATGGGCTCTGTCCG-3’. (III) HPV16E6-forward primer: 5’-ACTGCAATGTTTCAGGACCC-3’; reverse primer: 5’-GCATAAATCCCGAAAAGCAA-3’. The qPCR reaction was set up in a reaction volume of 20 μl. Each qPCR reaction contained, in 1 μl of total plasma DNA, 2 μl of 5 μM each forward and reverse primers, 10 μl of 2×Quantitect SYBR Green PCR Master Mix (Qiagen). For amplification of HPV16E6E7, the qPCR setting was initial denaturation at 95ºC for 5 min, followed by 45 cycles of denaturation at 95ºC for 10 s, annealing, and extension at 60ºC for 1 min. For amplification of HPV16E6 and HPV16E7, the qPCR setting was initial denaturation at 95ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for 10 s, annealing at 58ºC for 1 min, and extension at 72ºC for 1 min.

Determination of viral load

To prepare plasmids containing HPV16 E6 and/or E7 sequences for standard curves, we used the HPV16-positive cervical cancer cell line (Caski cell). Total cellular DNA was isolated by using Genomic DNA purification kit (Promega, Madison, WI) according to the instructions provided by the manufacturer. HPV16 E6 and/or E7 were recloned in PCR2.1-TOPO for standard curves. From the known molecular weights of the recombinant plasmids, the amount of plasmid DNA equivalent to 5×10^{10} copies of HPV16 E6 and/or E7 was first determined. Standard curves were generated automatically by plotting the threshold cycle values against the logarithm of the copy numbers of plasmid DNA standards (serial 10-fold dilution from 5×10^{10} to 0.5 copies

of HPV16 E6 and/or E7). The copy numbers of each sample was calculated from the standard curve. The concentration of plasma HPV DNA was expressed as copies of HPV genome per milliliter of plasma. The results are expressed as mean±SD (n=3).

Statistical analysis

The Mann-Whitney test was used to compare the HPV DNA copy numbers among cancers of different stages. Probability values <0.05 were regarded as statistically significant.

RESULTS

Quantitative PCR was highly sensitive in detection of HPV16 DNA with as little as 50 copies/μl. Plasma HPV16E6E7 DNA was detected in 6 of 20 (30.0%) patients with HPV16-positive invasive cervical cancer, but in none of those with CIN3 and normal controls (Table 2). The copy numbers of plasma HPV16 E6E7 DNA in patients with invasive cancer were higher than in microinvasive cancer, CIN3 and normal individuals (Table 2).

Table 3 shows the plasma HPV16E6E7 DNA viral loads, disease stage and serum levels of SCCA. Only four out of 13 (30.8%) patients with high serum SCCA levels had detectable levels of plasma HPV16E6E7 DNA before operation. On the other hand, two out of 7 (28.6%) patients with normal serum SCCA levels had detectable levels of plasma HPV16E6E7 DNA before operation.

We also checked plasma HPV16 DNA using other primers, including HPV16E7-forward-primer and HPV16E7-reverse-primer to detect part of E7 (70 bp) as well HPV16E6-forward-primer and HPV16E7-reverse-primer to detect part of E6 (159 bp), to rule out differences in HPV DNA detection rate in the same patient based on differences in primers' position (Table 3). HPV16E6 and/or E7 DNA were detected in the plasma of 2-3 out of 6 patients with cervical cancer who were positive for HPV16 DNA by the HPV16E6E7 primer.

Discussion

The major finding of the present study was the detection of HPV16 DNA in 60% of primary cervical cancers and in plasma samples of 30.0% of patients with HPV16 DNA-positive primary tumors.

The detection rate of HPV DNA in plasma of patients with uterine cervical cancer remains controversial. Pornthanakasem et al. (11) detected HPV DNA in the plasma of 12% of their HPV-positive patients with cervical cancer, whereas the same rate was 70% in another study (12). Furthermore, Yang et al. (13) reported detection of HPV16 DNA in plasma samples of 50% of their patients with cervical cancer. In this context, Capone et al. (15) reported that they could not detect HPV16 DNA in the plasma of 65 patients with nasopharyngeal SCC using L1 primer but detected the same DNA in the plasma of 2 of their 65 patients by real-time PCR using E7 primer. These differences in the detection rate may be due to differences in sample numbers and method of analysis.

In the present study, plasma HPV16E6E7 DNA was detected in 6 of 20 (30.0%) HPV16-positive patients with invasive cervical cancer but in none of the normal controls. The copy number of plasma HPV16 DNA in patients with invasive cancer was higher than in CIN3 and microinvasive cancer and in normal individuals. On the other hand, the copy number of plasma HPV16 E6E7 DNA in cervical cancer stage IVa was higher than that of cervical cancer stage IVb. More sample collection and further studies are required to determine the relationship between the detection level of HPV DNA in plasma and clinical stage of uterine cervical cancer.

The source of plasma HPV DNA remains unclear. However, the DNA level is probably related to tumor size, stage and presence or absence of metastasis. In this regard, Pornthanakasem et al. (11) reported that the plasma level of HPV DNA in metastatic patients was three times higher than that of patients without metastasis. Their results suggested that the amount of plasma HPV DNA could be a useful tumor marker for prediction of disease progression and clinical outcome after treatment of patients with cervical cancer.

In our study, only 30.8% of the patients with high levels of serum SCCA were plasma HPV16 DNA-positive. And detection rate of plasma HPV16 DNA was different based on differences in primers' position (Table 3). In this context, the form of HPV DNA in the plasma of cervical cancer patients is not clear. It is possible that various HPV DNA fragments are present in the peripheral circulation since the HPV DNA detection rates in the plasma varied with the use of different primers in the same patients with cervical cancer.

What is the source of HPV DNA in the peripheral circulation? While the exact source is not clear at this stage, circulating HPV DNA levels could represent lysis of circulating cancer cells or micrometastasis shed from the tumor (16, 17). Others have suggested that circulating tumor DNA in plasma might reflect tumor cell metastasis because of the high *in vitro* transforming activity (18, 19).

The results of the present study indicated that HPV16E6E7 DNA in plasma might not be a sensitive marker of cervical cancer recurrence because plasma HPV16 E6E7 DNA before operation was detected in only four out of 13 (30.8%) patients with high serum SCCA levels. However, HPV DNA could be potentially used as a marker of low-invasive cervical cancer tumors in patients with normal SCCA levels before treatment because two out of 7 (28.6%) patients with normal serum SCCA levels had detectable levels of plasma HPV16E6E7 DNA before operation. We recommend the use of several HPV DNA primers to detect various HPV DNA fragments in the peripheral circulation in patients with cervical cancer.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

(1) Kato H, Torigoe T. Radioimmunoassay for tumor antigen of human cervical squamous cell carcinoma. Cancer 1977;40:1621-1628.

(2) Murakami A, Suminami Y, Sakaguchi Y, Nawata S, Numa F, Kishi F, et al. Specific detection and quantitation of SCC antigen 1 and SCC antigen 2 mRNAs by fluorescence-based asymmetric semi-nested reverse transcription PCR. Tumour Biol 2000;21:224-234

(3) Mitsuishi K, Nakamura T, Sakata Y, Yuyama N, Arima K, Sugita Y, et al. The squamous cell carcinoma antigens as relevant biomarkers of atopic dermatitis. Clin Exper Allergy 2005;35:1327-1333

(4) Gutman LT, St Claire K, Herman-Giddens ME, Johnston WW, Phelps WC. Evaluation of sexually abused and non-abused young girls for intravaginal human papillomavirus infection. Am J Dis Child. 1992;146:694-699.

(5) Stevens-Simon C, Nelligan D, Breese P, Jenny C, Douglas JM Jr. The prevalence of genital human papillomavirus infections in abused and nonabused preadolescent girls. Pediatrics 2000;106:645-649.

(6) Kari S, Stima S. In: Infections in Human Pathology. pp. 117-126.

(7) Franco EL, Villa LL, Sobrinho JP, Prado JM, Rousseau MC, Desy M, et al. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. J Infect Dis. 1999;180:1415-1423.

(8) Kjaer SK, van den Brule AJ, Paull G, Svare EI, Sherman ME, Thomsen BL, et al. Type specific persistence of high risk human papillomavirus (HPV) as indicator of

high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. Br Med J 2002;325:572.

(9) Schlecht NF, Kulaga S, Robitaille J, Ferreira S, Santos M, Miyamura RA, et al. Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia. JAMA 2001;286:3106-3114.

(10) Cason J, Kaye JN, Jewers RJ, Kambo PK, Bible JM, Kell B, et al. Perinatal infection and persistence of human papillomavirus types 16 and 18 in infants. J Med Virol 1995;47:209-218.

(11) Pornthanakasem W, Shotelersuk K, Termrunglert W, Voravud N, Niruthisard S, Mutirangura A. Human papillomavirus DNA in plasma of patients with cervical cancer. BMC Cancer 2001;1:2

(12) Duenas A, Lizano M, Carrillo AL, Wojcik J, Trejo C. DNA of human papillomavirus detected by PCR in plasma of cervical cancer patients: A potential marker of residual disease. Clin Chem 2001;47:361-370

(13) Yang HJ, Liu VWS, Tsang PCK, Yip AMW, Tam KF, Wing LC, et al. Quantification of human papillomavirus DNA in the plasma of patients with cervical cancer. Int J Gynecol Cancer 2004;14:903-910

(14) Ho CM, Yang SS, Chien TY, Huang SH, Jeng CJ, Chang SF. Detection and quantitation of human papillomavirus type 16, 18 and 52 DNA in the peripheral blood of cervical cancer patients. Gynecol Oncology 2005;99:615-621

(15) Capone RB, Pai SI, Koch WM, Pai SI, Koch WM, Gillison ML, Danish HN, et al. Detection and quantitation of human papillomavirus (HPV) DNA in the sera of

patients with HPV-associated head and neck squamous cell carcinoma. Clin Cancer Res 2000;6:4171-4175.

(16) Pao CC, Hor JJ, Yang FP, Lin CY, Tseng CJ. Detection of human papillomavirus mRNA and cervical cancer cells in peripheral blood of cervical cancer patients with metastasis. J Clin Oncol 1997;15:1008-1012.

(17) Czegledy J, losif C, Hansson BG, Evander M, Gergely L, Wadell G. Can a test for E6/E7 transcripts of human papillomavirus type 16 serve as a diagnostic tool for the detection of micrometastasis in cervical cancer? Int J Cancer 1995;64:211-215.

(18) Anker P, Lyautey J, Lefort F, Lederrey C, Stroun M. Transformation of NIH/3T3 cells and SW480 cells displaying K-ras mutation. C R Acad Sci III 1994;317:869-874

(19) Garcia-Olmo D, Garcia-Olmo DC, Ontanon J, Martinez E, Vallejo M. Tumor DNA circulating in the plasma might play a role in metastasis: the hypothesis of the genometastasis. Histopathology 1999;4:1159-1164

Table 1. Background, treatment and prognosis of patients with cervical cancer.

Patient	Age	FIGO staging	Treatment	Pathology	Prognosis
CC1	20	Ia1	conization	SCC	CR
CC2	42	Ia1	Modified radical hysterectomy	SCC	CR
CC3	64	Ia1	Modified radical hysterectomy	SCC	CR
CC4	41	Ia1	Modified radical hysterectomy	SCC	CR
CC5	43	Ia2	Modified radical hysterectomy	SCC	CR
CC6	49	Ib1	Radical hysterectomy	SCC	CR
CC7	83	Ib1	Radiotherapy	SCC	CR
CC8	53	Ib2	Chemoradiotherapy	SCC	CR
CC9	42	Ib2	Radical hysterectomy*	SCC	CR
CC10	61	IIa	Chemoradiotherapy	SCC	CR
CC11	65	IIa	Radical hysterectomy**	SCC	CR
CC12	54	IIa	Radical hysterectomy***	SCC	CR
CC13	62	IIa	Chemoradiotherapy	SCC	CR
CC14	45	IIb	Chemoradiotherapy	SCC	CR
CC15	61	IIb	Not clear****	SCC	Not clear
CC16	60	IIb	Chemoradiotherapy	SCC	CR
CC17	56	IIb	Chemoradiotherapy	SCC	CR
CC18	80	IIIb	Radiotherapy	SCC	CR
CC19	65	IVa	Radiotherapy	SCC	PR
CC20	43	IVb	Chemoradiotherapy	SCC	PR

*Patient received chemoradiotherapy due to invasion of blood vessels after surgery. **Patient received chemoradiotherapy after operation.***Patient received Neo-adjuvant chemotherapy before operation.****Treatment and prognosis are not clear due to changes in hospitals. SCC: Squamous cell carcinoma. CR: Complete response to therapy and no recurrence or metastasis. PR: Partial Response

Table 2. Incidence of plasma human papillomavirus (HPV)16E6E7 DNA according to the stage of cervical cancers and detection levels of plasma HPV16E6E7 DNA in cervical cancer patients and precancerous disease.

	Cases	positive	Plasma HPV16E6E7 DNA (copies/ml of plasma)		
			Frequency (%)	maximum	median
Normal	20	0	0	0	0
NILM	3	0	0	0	0
L-SIL	1	0	0	0	0
H-SIL	20	0	0	0	0
Cancer (FIGO stage)					
Ia1	4	1	25	507	300
Ia2	1	0	0	0	0
Ib1	2	0	0	0	0
Ib2	2	0	0	0	0
IIa	4	1	25	96,583	96,583
IIb	4	2	50	34,2833	186,624
IIIb	1	0	0	0	0
IVa	1	1	100	229792	135381
IVb	1	1	100	17768	11935

Normal: negative control, NILM: negative for intraepithelial lesion or malignancy, HSIL: high-grade squamous intraepithelial lesion, LSIL: low-grade squamous intraepithelial lesion, FIGO: The International Federation of Gynecology and Obstetrics.

Table 3. Pre-treatment plasma HPV16 DNA levels and SCCA levels in HPV16-positive patients with cervical cancer.

Patient	Diagnosis	SCCA	(copies/ml of plasma)		
			HPV16E6E7	HPV16E7	HPV16E6
CC1	Ia1	2.1	0	0	0
CC2	Ia1	0.6	0	0	0
CC3	Ia1	0.9	0	0	0
CC4	Ia1	1.3	300	0	0
CC5	Ia2	1.7	0	0	0
CC6	Ib1	1.1	0	0	0
CC7	Ib1	1.3	0	0	0
CC8	Ib2	14.3	0	0	0
CC9	Ib2	9.9	0	0	0
CC10	IIa	3.0	96583	334000	0
CC11	IIa	2.6	0	0	0
CC12	IIa	2.7	0	0	0
CC13	IIa	1.7	0	0	0
CC14	IIb	12.9	0	0	0
CC15	IIb	9.3	342833	129000	1190000
CC16	IIb	18.2	0	0	0
CC17	IIb	1.5	30417	406000	556667
CC18	IIIb	1.4	0	0	0
CC19	IVa	35.9	135381	0	0
CC20	IVb	12.2	11935	0	0