RESEARCH ARTICLE

Prognostic Value of HPV18 DNA Viral Load in Patients with Early-Stage Neuroendocrine Carcinoma of the Uterine Cervix

Sumalee Siriaunkgul[1], Utaiwan Utaipat[2], Supaporn Suwiwat[3], Jongkolnee Settakorn[1], Kornkanok Sukpan[1], Jatupol Srisomboon[4], Surapan Khunamornpong[1]*

Abstract

Objectives: To evaluate the clinicopathologic correlation and prognostic value of HPV18 DNA viral load in patients with early-stage cervical neuroendocrine carcinoma (NECA). Methods: Formalin-fixed, paraffin-embedded tissue of cervical NECA patients with known HPV18 infection and clinicopathologic data including follow-up results were collected. The HPV18 DNA load was assessed with quantitative PCR targeting the HPV18 E6E7 region. Results: Twenty-one patients with early-stage (IB-IIA) cervical NECA were identified. HPV18 DNA viral load ranged from 0.83 to 55,174 copies/cell (median 5.90). Disease progression, observed in 10 cases (48%), was not significantly associated with any clinicopathologic variables. However, the group of patients with progressive disease tended to have a higher rate of pelvic lymph node metastasis (50% versus 9%, p=0.063) and a lower median value of HPV18 DNA viral load (4.37 versus 8.17 copies/cell, p=0.198) compared to the non-recurrent group. When stratified by a cut-off viral load value of 5.00 copies/cell, the group of patients with viral load ≤5.00 copies/cell had a significantly shorter disease-free survival than the group with viral load >5.00 copies/cell (p=0.028). The group with a lower viral load also tended to have a higher rate of disease progression (75% versus 31%, p=0.080). No significant difference in the other clinicopathologic variables between the lower and higher viral load groups was identified. Conclusion: HPV18 DNA viral load may have a prognostic value in patients with early-stage NECA of the cervix. A low viral load may be predictive of shortened disease-free survival in these patients.

Keywords: Human papillomavirus (HPV) - HPV18 - DNA viral load - prognosis - neuroendocrine carcinoma

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Introduction

Cervical carcinoma is associated with human papillomavirus (HPV) infection (Woodman et al., 2007). Squamous cell carcinoma and adenoarcinoma are the most common subtypes of cervical cancer and comprise the large majority of cases. Neuroendocrine carcinoma (NECA) is a rare and aggressive subtype of cervical carcinoma with a worse prognosis than the other subtypes (Wells et al., 2003). The distribution of HPV types is variable in different subtypes of cervical carcinoma. In squamous cell carcinoma, HPV16 is the predominant type detected in almost 60% of cases, whereas HPV18 infection is much less common (Siriaunkgul et al., 2008). In adenocarcinoma, HPV18 infection is detected in a significant proportion of cases, which is only slightly lower than that of HPV16 infection. In NECA, HPV18 is the most prevalent HPV type, found in 75% of cases (Siriaunkgul et al., 2011).

In addition to the identification of HPV DNA, quantitative measurement of the viral load is also another interesting issue concerning the relationship between HPV infection and cervical neoplasia. The clinical significance of HPV viral load in patients with cervical carcinoma has been evaluated in only a small number of studies (Datta et al., 2006; De Boer et al., 2007; Kim et al., 2008: 2009; Gnanamony et al., 2009; Kang et al., 2011). An association between a high HPV viral load in cervical carcinoma and a better prognosis has been described (Datta et al., 2006; Kim et al., 2009). To our knowledge, a study on HPV viral load in cervical NECA has not been reported.

The aim of this study was to evaluate the HPV18 DNA viral load in patients with early-stage cervical NECA and its possible clinicopathologic correlation.

Materials and Methods

This study was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University. The cases of cervical NECA diagnosed between 2003-2009, which were known to be HPV18-positive in a previous PCR study (Siriaunkgul et al., 2011), were retrieved. The diagnosis of NECA was based on the standard morphologic criteria by a consensus agreement of 4 pathologists (S.S., S.K., J.S, and K.S.), with further classification into atypical carcinoid, small cell carcinoma, and large cell NECA

*[1]Department of Pathology, Faculty of Medicine, [2]Research Institute for Health Sciences, [4]Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Chiang Mai, [3]Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Songkhla, Thailand *For correspondence: skhunamo@med.cmu.ac.th*

(Albores-Saavedra et al., 1997; Wells et al., 2003). The diagnoses of atypical carcinoid and large cell NECA required a confirmation by a positive immunoreaction for at least one of the following neuroendocrine markers: chromogranin A, synaptophysin, or CD56. The clinical data and follow-up information was obtained from the chart review. Tumor staging was based on the International Federation of Gynecology and Obstetrics (FIGO) staging system. The patients with early-stage tumors (IB-IIA) were treated with surgery, followed by adjuvant chemotherapy (cisplatin 75 mg/m^2 and etoposide 100 mg/m^2), with or without radiation therapy.

To evaluate the clinicopathologic and prognostic correlation of HPV18 DNA viral load, only the cases with follow-up results were included in this study. One formalin-fixed, paraffin-embedded tissue block from each case was selected for the viral load measurement and HPV in situ hybridization (ISH). For the viral load study, the non-neoplastic part of the block was trimmed out to obtain the sections containing NECA as an almost exclusive component of the samples.

DNA extraction from paraffin-embedded tissue was performed based on previously described methods (Siriaunkgul et al., 2011). Quantiative PCR (qPCR) for HPV18 quantitation was carried out using TaqMan chemistry on the ABI PRISM 7500 Sequence Detection System instrument. The reaction was set up as previously described by Gravitt et al. 2003, with minor modifications to accommodate with the machine platform and the chemical components. The HPV18 DNA load was performed targeting the 184-bp of the E6E7 region using the primers 18U648: AAATTCCGG TTGACCTTCTA and 18L811: GTCTGCTGAGCTTTCTACTACT and the TaqMan probe 18UTQ1: HEX-CGTCGG GCTGGTAAATGTTGATG-BHQ1. The qPCR was set up in a 50-μL final volume containing 20mM Tris-HCl pH 8.4, 50mM KCl, 4mM $MgCl_2$ (diluted from 10X PCR buffer and 50mM $MgCl_2$ supplied with Platinum *Taq* Polymerase), 200 nM of each dNTPs, 25 pmol of each primers, 20 pmol of probe, and 3U of Platinum *Taq* Polymerase (Invitrogen) in the presence of 0.3 μL of 1:100X ROX dye (Finnzymes, Vantaa, Finland). The thermal profile was 95°C 10 min; 50 cycles of 95°C 15 sec, 55°C 33 sec. Samples were amplified in duplicate using a 2.5 μL of undiluted and at a 1:10 dilution of DNA extracts. Final viral loads were quantified utilizing the undiluted samples in case of the expected 10-fold of undiluted: diluted ratio of each sample was obtained and the viral load based on the diluted sample was utilized when the PCR inhibitor was evident in the amplification. The qPCR using plasmid containing full-length HPV18 genome (gift from Dr. Keerti V. Shah, Johns Hopkins University) was performed along with the samples at the amount of 10, 10^2, 10^3, 10^4, 10^5 and 10^6 copies to generate a standard curve with the slopes that ranged from -3.372 to -3.520, and the de-ionized water in place of DNA was used as a no target control. For cellularity normalization, the human β globin gene qPCR was carried out in all samples with similar reaction condition described above using the previously described primers (Ersahin et al., 2005) globin sense; ACACAACTGTGTTCACTAGC, globin anti-sense; TTCTCTGTCTCCACATGCCC flanking the designed Taqman globin probe: Cy5-TCCTGAGGAGAAGTCTGCCGTTACT-BHQ2. The qPCR of β-globin gene on the DNA extracts from SiHa cell line (containing HPV16 at 1-2 copies per cell) from the ten-fold dilution starting from 10^7 cells was used as a standard curve with the slopes ranged from -3.594 to -3.668. The ratio Ct of HPV16 and β-globin of ~ 1.04 was obtained in all SiHa cell dilutions indicated the equivalent amount of these 2 genomes in the SiHa cell (data not shown). The normalized HPV18 DNA viral load values expressed as viral copies per cell (equivalent to 2 copies of β-globin gene) were calculated using the following method: normalized viral load = [HPV copies number/ β-globin gene copy number] × 2.

The correlation between the clinicopathologic variables and clinical outcome was evaluated by the Fisher's exact test or t-test as appropriate. The association between the viral load and the survival was estimated by Kaplan-Meier survival curve and log-rank test for equality of survivor functions. p value <0.05 was considered as statistically significant.

For ISH, 5-micron sections were cut from each paraffin block and deparaffinized with xylene. The sections were pretreated with sodium citrate pH 6.0 at 90ºC for 15 min and digested with pepsin 0.1% at 37ºC for 15 min. Endogenous peroxidase blocking was performed using 1% hydrogen peroxide for 15 min. A biotinylated probe for HPV16/18 (PathoGene HPV type16/18 probe, Enzo Life Science, UK) was added to the sections, heated at 90ºC for 5 min, and left overnight in a humidity chamber at 37ºC. The signal was detected by streptavidin-biotin system with tyramide signal amplification, using DAB as a chromogen substrate (GenPoint™ Tyramide Signal Amplification System for Biotinylated probes Kit, DAKO) The signals in the nuclei of neoplastic cells were classified as punctuate type or diffuse type or combined type (Cooper et al., 1991).

Results

Twenty-six cases of cervical NECA with previously known HPV18 infection were retrieved, composed of atypical carcinoid (2 cases), small cell carcinoma (21 cases), and large cell NECA (3 cases). Mixed components of other subtypes of invasive carcinoma were present in 6 cases, including adenocarcinoma in 5 and squamous cell carcinoma in 1 case. The patients' ages ranged from 25 to 61 years (mean±SD 41.6±8.7). The FIGO tumor stage was IB1 in 12 cases, IB2 in 7 cases, IIA in 2 cases, IIB in 3 cases, IIIB in 1 case, and IVB in 1 case. The follow-up duration ranged from 4 to 92 months (mean±SD 36.2±25.6, median 28.5). Co-infection with HPV16 was present in 5 cases. The normalized HPV18 DNA viral load ranged from 0.83 to 55, 174.96 copies/cell (median 7.06). There was no significant difference in the median values of HPV18 DNA viral load between cases with early stage (IB-IIA, 21 cases) and advanced stage (IIB-IVB, 5 cases) (Table 1).

Twenty-one cases were of early stage and were treated by surgery with adjuvant therapy. Disease progression

Table 1. The Characteristics of Patients with Cervical Neuroendocrine Carcinoma in Early Stage (IB-IIA) and Advanced Stage (IIB-IV)

	Early stage (n=21)	Advanced stage (n=5)	P value
Mean age (yr±SD)	41.5±9.4	45.4±6.5	0.392[T]
Histology			
Atypical carcinoid	2	0	0.691[F]
Small cell	17	4	
Large cell	2	1	
Range of viral load*	0.83-55,174	2.20-229	-
Median viral load*	5.9	93.8	0.143[T]

*copies/cell, [F]Fisher Exact test, [T]T-test

Table 2. Comparison of the Clinicopathologic Features between Patients with and Without Disease Progression of Early-Stage Cervical Neuroendocrine Carcinoma.

	No recurrence n=11 (%)	Disease progression n=10 (%)	P value*
Mean age (yr±SD)	40.9±8.4	42.2±10.7	0.761[T]
Stage IB1	7 (64)	5 (50)	0.817
IB2	3 (27)	4 (40)	
IIA	1 (9)	1 (10)	
Histology of NECA			
Atypical carcinoid	2 (18)	0 (0)	0.228
Small cell	9 (82)	8 (80)	
Large cell	0 (0)	2 (20)	
Mixed component of other carcinoma subtype	3 (27)	2 (20)	>0.999
Lymph node metastasis	1 (9)	5 (50)	0.063
Co-infection with HPV16	2 (18)	3 (30)	0.635
Median of HPV18 viral load	8.17	4.37	0.198[T]
Median follow-up duration (mo)	63	14	0.002[T]

*p value by Fisher Exact test if not otherwise specified

was observed in 10 cases (48%), all except one of these cases developed distant metastasis. The remaining case had pelvic recurrence followed by distant metastasis. Comparison of the clinicopathologic variables and HPV18 DNA viral load between cases without recurrence and cases with disease progression is shown in Table 2. The group with disease progression had a higher rate of pelvic lymph node metastasis compared to the non-recurrent group (50% versus 9%), with a marginal significance of the difference (p=0.063). The median value of HPV18 DNA viral load was lower in the group with disease progression than in the non-recurrent group (4.37 versus 8.17 copies/cell), but the difference was not significant. The group with disease progression had a significantly shorter median follow-up duration because most patients were lost to follow-up after disease progression and treatment failure.

Table 3 shows a comparison of clinicopathologic variables and HPV18 DNA viral load between 2 groups of patients stratified by a cut-off viral load value of 5.00 copies/cell. There was no significant difference between both groups in tumor stage, histology, extent of involvement, and pelvic lymph node metastasis. The group with viral load ≤5.00 copies/cell tended to have a higher progression rate (75% versus 31%) and shorter

Table 3. Comparison of the Clinicopathologic Features of the Patients with Early-Stage Cervical Neuroendocrine Carcinoma Stratified by HPV18 DNA Viral Load

	Viral load (copies/cell) ≤5.00, n=8 (%)	>5.00, n=13 (%)	P value*
Mean age (yr±SD)	44.6 ±8.4	39.6 ±9.8	0.244[T]
Stage IB1	5 (62)	7 (54)	0.83
IB2	2 (25)	5 (38)	
IIA	1 (13)	1 (8)	
Histology			
Atypical carcinoid	1 (12)	1 (8)	0.757
Small cell	7 (88)	10 (77)	
Large cell	0	2 (15)	
Invasion to the outer third of wall	5 (63)	9 (69)	>0.999
Parametrial involvement	2 (25)	7 (54)	0.367
Lymphovascular invasion	8 (100)	10 (77)	0.257
Lymph node metastasis	3 (38)	3 (23)	0.631
Co-infection with HPV16	2 (25)	3 (23)	>0.999
Disease progression	6 (75)	4 (31)	0.08
Median disease-free duration (mo)	16	53	0.183[T]

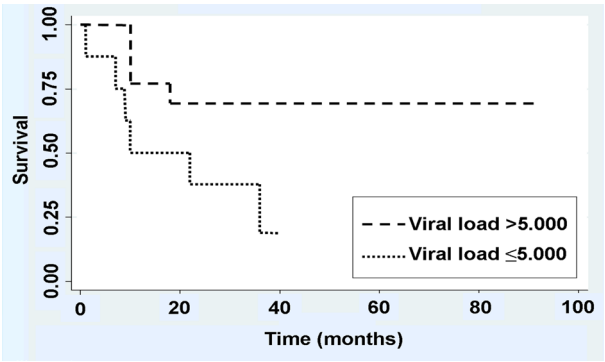

Figure 1. Kaplan-Meier Plot of HPV18 DNA Viral Load and Disease-Free Survival in Patients with Early-Stage Cervical Neuroendocrine Carcinoma.

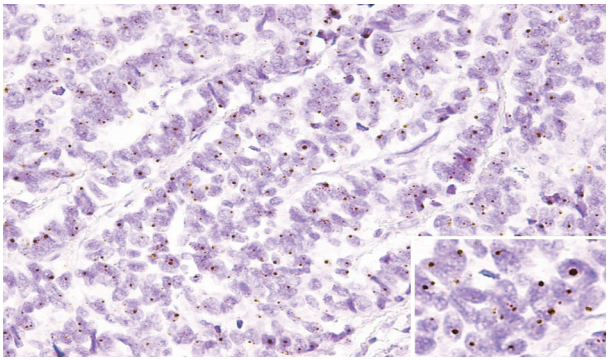

Figure 2. HPV in Situ Hybridization in Cervical Small Cell Carcinoma. Punctate signals in the nuclei of neoplastic cells were observed (inset).

median disease-free duration (16 versus 53 months) but the differences were not statistically significant. Analysis of disease-free survival by Kaplan-Meier plot revealed a significantly shorter disease-free survival in the group with viral load ≤5.00 copies/cell (p=0.028) (Figure 1). The analysis for overall survival was not possible due to the loss to follow-up of the patients who failed treatment.

In 5 patients with advanced stage NECA, one stage

IIB patient was free of tumor recurrence up to 40 months of follow-up duration, whereas the remaining 4 cases had progressive disease with distant metastasis. The DNA viral load in this non-recurrent case was 115.97 copies/cell.

ISH by HPV16/18 probe showed only punctuate signals within the nuclei of neoplastic cells in 25 of 26 cases of NECA of all stages (Figure 2). In the remaining case with DNA viral load of 33.98 copies/cell, no signals were detected although repeat ISH was performed.

Discussion

Viral load in neoplasms that are pathogenetically associated with viral infection received an interest whether it may provide useful information on neoplastic progression or as a possible prognostic marker. The relationship between the viral load value and the clinical outcome may be variable based on the type of viruses and the type of associated neoplasms. In hepatocellular carcinoma, a low serum viral load of hepatitis B virus DNA at the diagnosis was associated with an improved survival rate of the patients (Ohkubo et al., 2002). In nasal NK/T-cell lymphoma, a low viral load of Epstein-Barr virus detected in formalin-fixed paraffin-embedded tissue was reported to be associated with better overall survival of the patients, compared to the high viral load (Hsieh et al., 2007). The prognostic value of HPV viral load has been evaluated in studies on HPV-associated cancers such as tonsillar squamous cell carcinoma and cervical carcinoma (Mellin et al., 2002; Datta et al., 2006; de Boer et al., 2007; Cohen et al., 2008; Kim et al., 2008: 2009; Gnanamony et al., 2009; Kang et al., 2011). The studies on HPV16 DNA viral load detected in tissue samples (fresh frozen or paraffin-embedded) of tonsillar carcinoma demonstrated a significant association between low viral load and adverse prognosis (Mellin et al., 2002; Cohen et al., 2008).

In cervical carcinoma, viral load studies were based on different types of material such as cervical scrape (Datta et al., 2006; Kim et al., 2008: 2009; Cheung et al., 2009; Kang et al., 2011), fresh frozen tissue (Gnanamony et al., 2009), or formalin-fixed paraffin-embeded tissue (De Boer et al., 2007; Yoshida et al., 2009). Although DNA fragmentation and lower DNA yield are limitations for PCR study on formalin-fixed paraffin-embedded tissue, this is the type of sample that could be most practically obtained in a retrospective analysis or after a histologic examination has been completed. The prognostic significance of HPV viral load in cervical carcinoma has been evaluated in only a few studies. Low HPV viral load by Hybrid Capture II test in cervical specimens from patients with cervical carcinoma treated by radiation therapy was an independent prognostic predictor of poor clinical outcome (Datta et al., 2006; Kim et al., 2009), although the clinical significance of HPV viral load was not confirmed in other studies (De Boer et al., 2007; Kim et al., 2008; Kang et al., 2011). In the study by de Boer et al (De Boer et al., 2007), a high expression of E6/E7 mRNA of HPV16 or HPV18 was a poor prognostic predictor in surgically-treated patients with cervical carcinoma of non-NECA type, whereas the DNA viral load was not. It may be uncertain whether the variation in sample types or study methods would affect the assessment of the clinical significance of HPV viral load in cervical carcinoma.

Although HPV infection is a necessary cause in the development of cervical carcinoma, the natural history of HPV infection in the cervix has not been completely understood (Woodman et al., 2007). The association between the viral load, the physical status of viral genome, the type or degree of epithelial lesion, and the type of HPV is a complex and unresolved issue (Woodman et al., 2007; Cheung et al., 2009). The significance of the viral load may vary with different types of HPV infection. For example, while HPV16 viral load was increased with the severity of lesions from precancerous lesion (squamous intraepithelial lesion) to invasive carcinoma, HPV18 viral load did not correlate with the severity of disease (Woodman et al., 2007; Saunier et al., 2008; Xi et al., 2009).

In this study, we focused on the HPV18 DNA viral load because this is the major type of HPV infection in cervical NECA. The patients without tumor recurrence tended to have a lower rate of lymph node metastasis and higher viral load compared to those with disease progression. Using the cut-off viral load value of 5.00 copies/cell, the patients with a higher viral load showed a significantly better disease-free survival as compared to those with a lower viral load. The rate of disease progression also tended to be lower in the group with a higher viral load (31% versus 75%). Although a multivariate analysis was not possible due to the small number of cases in this study, there was no apparent tendency or association of HPV18 DNA viral load with other standard adverse prognostic features such as the local extent of tumor invasion or lymph node metastasis (Table 3). In 2 previous studies on cervical carcinoma of non-NECA subtypes, HPV18 DNA viral load was also not associated with tumor stage or lymph node metastasis (De Boer et al., 2007; Gnanamony et al., 2009).

In general, a low viral load seems to be associated with the integrated physical status of HPV genome in which viral replication is unlikely, whereas the presence of episomal HPV genome may be related to the high viral load (Woodman et al., 2007; Cheung et al., 2009). In this study, only the punctuate type of ISH positive signals, which represented the integrated physical status of HPV (Cooper et al., 1991), was detected in almost all specimens, whereas a diffuse or combined ISH pattern which indicated the presence of episomal HPV was not observed.

The underlying mechanism for possible association of low viral load and poor prognosis in cervical carcinoma is not clear. In patients with cervical cancer, there are complex alterations that suppress immune response to cancer cells, such as an impaired response of cytotoxic lymphocytes, an increase of regulatory T cells, or defective T cell signaling (Patel and Chiplunkar, 2009). The possibility that a high viral load may be associated with increased HPV antigen expression, resulting in some improvement of host immune response against the neoplastic cells, could be an explanation for a favorable outcome in patients with HPV-related cancers (Datta et al., 2006; Cohen et al., 2008; Kim et al., 2009). In support of this view, HPV E6 and E7 antigens are currently the targets

for the development of therapeutic vaccines for cervical carcinoma (Su et al., 2010). If the postulation could be true in patients with early-stage cervical NECA, an increased immune response against the neoplastic cells may promote host defense mechanisms in the control tumor metastasis or may enhance the effect of postoperative adjuvant chemotherapy or radiation therapy in the elimination of residual microscopic tumor cells that have spread beyond the uterus, thus reducing the chance of disease progression. Further studies are necessary to clarify the prognostic role of HPV18 DNA viral load in patients with cervical NECA.

In conclusion, HPV18 DNA viral load may have a prognostic value in patients with early-stage NECA of the cervix. A low viral load may be predictive of shortened disease-free survival in these patients.

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