Differences in Human Papillomavirus Type May Influence Clinical Outcome in Early Stage Cervical Cancer

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Abstract. *Background: The presence of human papillomavirus (HPV), the HPV type and viral load in early stage cervical carcinoma were investigated in order to elucidate whether any of these factors were important for clinical outcome. Patients and Methods: Twelve patients who were disease-free 5 years after diagnosis were matched and compared with 12 patients who died within 2 years. The presence of HPV, HPV type and viral load in their tumours was examined by PCR. Results: The distribution and load of HPV was similar in the 2 patient groups. HPV-16 was, however, significantly more common in tumours of the surviving patients than in those of patients who died (88.9% and 18.2%, respectively, p=0.0152). Conclusion: HPV-16 was significantly more common in early stage carcinomas of patients surviving more than 5 years in comparison to early stage carcinomas of patients with a poor prognosis.*

Cervical carcinoma is the second most common malignant disease in women and each year 510,000 new cases are diagnosed and 288,000 women die from the disease worldwide (http://www.who.int/vaccine_research/diseases/hpv/en/). Persistent infection with high-risk types of human papillomavirus (HPV), especially types -16 and -18, is regarded as the principal risk factor in the development of squamous cervical cancer (1, 2). Most HPV infections regress spontaneously, however, some infections can, after a long latency, cause cancer. Viral load has been suggested to be a marker of persistent infection (3) and it has been shown that viral load is of importance for the risk of developing a pre-cancerous lesion (4) as well as for the progression of a pre-cancerous lesion into a carcinoma (5-7). Furthermore, in tonsillar carcinoma, the presence of HPV-16 and a high viral load in the tumour tissue have been shown to be favourable prognostic factors (8). The aim of this study was to investigate if the presence of HPV, the HPV type and viral load had any impact on clinical outcome in early stage cervical carcinoma stage Ib and IIa patients, where the overall survival is generally around 70-90% (9). This was accomplished by comparing the presence of HPV, the HPV type and viral load in paraffin-embedded tumour biopsies from 12 patients who were disease-free 5 years after diagnosis, with tumour biopsies from 12 patients who died from their disease within 2 years after diagnosis.

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Key Words: Cervical neoplasia, cervical squamous cell carcinoma, papillomavirus.

Patients and Methods

Patients and tumour biopsies. Twenty-four patients, treated for cervical carcinoma stage Ib or IIa at the Department of Gynaecological Oncology, Radiumhemmet, Karolinska University Hospital, Sweden, between 1994 and 1997, were identified and included in the study. The records and pathology materials were reviewed retrospectively, both clinically and pathologically, by one gynaecological oncologist and one pathologist. The material was divided into 2 groups according to patient survival time, *i.e.* 12 patients surviving more than 5 years and 12 patients dying within 2 years after diagnosis. The material was matched as well as practically possible with regard to tumour stage, histology, tumour size, grade, treatment, lymph node status and age at diagnosis (Table I). Other variables such as smoking habits, parity and usage of hormonal replacement therapy were also matched between the 2 groups. During the study period, 2 cases in separate pairs had to be excluded because the original slides were of poor quality and a new matching procedure (pairs 11 and 12) had to be performed.

Tumour biopsies were collected at the time of diagnosis, fixed in formalin and paraffin embedded. Eight sections, each 5 μm thick, were cut and collected in a tube for DNA extraction. DNA was extracted from 8x5 μm tumour sections according to the manufacturer's instructions for the High Pure RNA Paraffin Kit (Roche) with exclusion of the DNase treatment.

Table I. *Summary of matching data in cervical carcinoma divided into short- and long-time survivors (n=24), according to age, stage and histology.*

Survival	>5 years (n=12)			<2 years (n=12)		
Pair[1]	Age[2]	Stage	PAD[3]	Age	Stage	PAD
1	49	Ib2	squamous cell carcinoma	48	Ib2	squamous cell carcinoma
2	35	Ib1	squamous cell carcinoma	37	Ib1	squamous cell carcinoma
3	48	Ib1	squamous cell carcinoma	49	Ib1	squamous cell carcinoma
4	42	Ib1	squamous cell carcinoma	42	Ib2	squamous cell carcinoma
5	41	Ib1	squamous cell carcinoma	36	Ib1	squamous cell carcinoma
6	40	Ib2	adenocarcinoma	39	Ib1	adenocarcinoma
7	33	Ib2	adenosquamous	30	IIa	adenosquamous
8	56	IIa	adenocarcinoma	57	IIa	adenosquamous
9	35	IIa	adenosquamous	36	IIa	squamous cell carcinoma
10	31	Ib1	adenosquamous	32	Ib2	squamous cell carcinoma
11	42	Ib1	adenocarcinoma	38	Ib1	squamous cell carcinoma
12	49	Ib1	squamous cell carcinoma	50	Ib1	adenocarcinoma

[1]Matched patients; [2]Age at diagnosis; [3]Pathology at diagnosis.

Detection and quantification of HPV – study design. In cervical carcinoma in Sweden, HPV-16 is the most common HPV type, followed by HPV-18 and hence, for the sake of simplicity, all samples were quantified for HPV-16 first (see below). If the samples were negative for HPV-16, they were tested for the presence of HPV with consensus primers. HPV-positive samples were quantified for HPV-18 and those that were negative were sequenced as described below. HPV-16 and HPV-18 were quantified in triplicates of 0.1 ng, 1 ng and 5 ng sample DNA. For biopsies with such a low viral load that the signal was weaker than for the standard with the lowest number of HPV plasmid copies, a 10 ng sample DNA was run in a new run. The median value of HPV copies per cell was calculated from 2 to 3 dilutions.

HPV-16 and HPV-18 quantification. To estimate the number of HPV-16 copies per cell, a quantitative real-time PCR method (TaqMan) based on the 5'-3' exonuclease activity of Taq DNA polymerase was used (8). The 25 μl PCR volume consisted of 12.5 μl TaqMan® Universal PCR Master Mix (Applied Biosystems), 7.5 pmol of each primer, 2.5 pmol of the probe, 1x Rox Reference Dye (Invitrogen™) and one of 3 amounts of the template (0.1 ng, 1 ng or 5 ng). The PCR was carried out in an iCycler iQ real-time detection system (BioRad) with an initial step of 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 15 sec at 55˚C and 1 min at 60˚C. All cervical cancer samples were run in triplicate in parallel with the standard dilutions of the HPV-16 plasmid (8).

To estimate the number of HPV-18 copies per cell, a quantitative real-time PCR method based on the SYBRGreen system was used. In order to create a standard with a known amount of virus copies, cloned HPV-18 was series diluted and used in each real-time assay as a standard to calculate the number of viral copies. The PCR volume of 25 μl consisted of 12.5 iQ™ SYBRGreen supermix (BioRad), 1.25 μl (10 pmol/μl) each of the HPV-18 primers (10) and one of the amounts of the template (0.1 ng, 1 ng or 5 ng and, in some cases, 10 ng sample DNA). The PCR was carried out in an iCycler iQ real-time detection system (BioRad) with an initial step of 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 30 sec at 57.7˚C and 1 min at 74˚C. All cervical cancer samples were run in triplicate in parallel with the standard dilutions of the HPV-18 plasmid.

Table II. *Comparison of HPV status and viral load between matched patients.*

Survival	>5 years (n=12)		<2 years (n=12)	
Pair	HPV status	Viral load[1]	HPV status	Viral load
1	HPV-16	0.005	HPV-16	0.007
2	HPV-16	0.26	HPV-16	0.2
3	HPV-16	0.09	HPV-18	0.07
4	HPV-16	0.07	HPV-18	36.9
5	Negative	Not done	Negative	Not done
6	HPV-16	0.02	HPV-45	Not done
7	Negative	Not done	Negative	Not done
8	Negative	Not done	Negative	Not done
9	HPV-16	3.5	HPV-18	0.04
10	HPV-18	0.02	Positive[2]	Not done
11	HPV-16	0.002	HPV-18	0.06
12	HPV-16	0.38	HPV-18	0.02
	9/12 HPV pos		9/12 HPV pos	

[1]Viral load was determined as copies/cell and presented as the median value calculated from 2 to 3 dilutions.
[2]This sample was HPV-positive, but could not be type determined, however it was not HPV-16- or -18-positive.

Detection of HPV. Samples which were negative in the HPV-16 type-specific real-time PCR were run in a broad spectrum HPV PCR, with GP5+/6+ primers to detect other HPV types (8, 11), and samples which were negative using these primers were run using consensus primers CPI/IIG (8, 12). To rule out false-negative results, the HPV-negative samples were assayed with S14 primers (13).

HPV typing. HPV sequencing was performed by direct sequencing of the purified PCR products from the GP5+/6+ primers using the Big Dye Terminator Cycle Sequencing Kit, in an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Both DNA strands were sequenced and aligned to those available at NCBI BLAST GenBank (www.ncbi.nlm.nih.gov/BLAST/). In addition, the samples were also run with the CPI/IIG primers and one of the strands was sequenced as described above.

Results

Presence of HPV, HPV type and clinical outcome. Eighteen out of the 24 patients had HPV-positive tumours (75.0%). Ten samples were HPV-16-positive (by HPV-16 quantitative PCR). Six samples were shown to be positive for HPV-18 (by HPV-18 quantitative PCR) and one for HPV-45 (by general primers and sequencing). The final sample was not possible to sequence; it was negative in both the HPV-16 and -18 type-specific PCRs, but positive by general HPV PCR.

Nine patients in each group (75.0%) were HPV-positive (Table II). Within the group of patients who survived more than 5 years, 8 were HPV-16-positive (88.9%) and one was HPV-18-positive (11.1%). Among the group of patients who died within 2 years after diagnosis, 2 were HPV-16-positive (22.2%), 5 were HPV-18-positive (55.6%), one was HPV-45-positive (11.1%) and one was not possible to type; this last sample was, however, negative for both HPV-16 and -18. HPV-16 was significantly more common in tumours of the surviving patients as compared to tumours of patients who died within 2 years after diagnosis ($p=0.052$, Fisher's exact). HPV-18 was more common in these patients (5/12) compared to the patients (1/12) who survived for more than 5 years, but this difference was not statistically significant.

HPV viral load and clinical outcome. The median value of HPV-16 and -18 copies per cell, calculated from 2 to 3 dilutions, is shown for the matched patients in Table II. The viral load for HPV-16 ranged between 0.002-3.5 copies/cell and did not differ between the patient group alive 5 years after diagnosis (0.002-3.5 copies/cell, n=8) and the group who died within 2 years after diagnosis (0.007-0.2 copies/cell, n=2). The viral load for HPV-18 ranged between 0.02-36.9 copies/cell and did not differ between the patients who were still alive 5 years after diagnosis (0.02 copies/cell, n=1) and the group who died within 2 years after diagnosis (0.02-36.9 copies/cell, n=5).

Discussion

It has been concluded that the HPV load is a type-dependent risk marker for invasive cervical carcinoma (14) and that there is an association between a high viral load and persistence of infection with HPV (3). Furthermore, Andersson *et al.* (15) have recently estimated the viral load of high-risk HPV in a systemic manner in pre-stages of cervical cancer (CIN). They found a wide range of viral load values, overall several magnitudes, regardless of CIN grade, but the average HPV copy number per cell was not significantly different between the CIN I-II-III groups.

In this study, the possible influence of the presence of HPV, the HPV type and viral load on clinical outcome was investigated in stage Ib and IIa cervical carcinoma. Twelve patients who were still alive 5 years after diagnosis and 12 patients who died within 2 years from diagnosis were selected and matched regarding age at the time of diagnosis, tumour stage and grade and lymph node status. Although 75% of the tumours from patients in both groups were HPV-positive, the type distribution was somewhat different between the 2 groups. HPV-16 was significantly more common in the tumours of patients who survived for more than 5 years after diagnosis than in the tumours from the patient group who died within 2 years. In contrast, although not statistically significantly, HPV-18 was more common in the patients who died within 2 years as compared to the patient group with the better prognosis. Nevertheless, both these results must be taken with some caution since the number of cases was limited.

The HPV viral load was similar in the tumours of the 2 groups and hence could not be used as a marker of prognosis. These results differed when compared to those of a study on tonsillar cancer, where a high HPV-16 viral load was a favourable prognostic factor (8). The distribution of HPV-16 differed between the 2 groups, as mentioned above. Furthermore, notably, the HPV-16 viral load was higher in the tonsillar cancer (8) study (10 – several thousands copies/cell) compared to that in cervical cancer in the present study (0.002-3.5 copies/cell). This was true even when taking into account that fresh frozen tonsillar cancer samples were analyzed in the former study and paraffin-embedded cervical cancer samples were analyzed here and that a higher viral copy number is generally obtained in fresh frozen material (Dahlgren, unpublished data). The reason for the discrepancy in viral load between tonsillar and cervical cancer is unknown, but could be due to the fact that the tumours are located and develop differently, and should be studied further.

In conclusion, HPV-16 was significantly more common in the tumours of patients with cervical cancer stage Ib and IIa who survived for more than 5 years after diagnosis as compared to tumours of patients with similar stage cervical cancer who died. There was also a tendency for HPV-18 to be more common in the tumours of patients with cervical cancer stage Ib and IIa who died within 2 years as compared to the tumours of patients with similar stage cancer who survived. An expanded study to confirm these findings would be of value. The HPV viral load was similarly independent of clinical outcome.

Acknowledgements

The authors would like to thank the Swedish Cancer Foundation, the Swedish Research Council, the Stockholm Cancer Society, the Stockholm City Council and the Karolinska Institute, Sweden, for financial support.

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Received November 29, 2005
Accepted January 17, 2006