A perspective study on correlation between HPV DNA and lymph nodes in surgically treated cervical carcinoma patients. Preliminary data

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Summary

The purpose of this study was to analyze the presence of HPV DNA in lymph nodes in patients with cervical cancer. *Study Design:* A prespective study was performed on a total of 18 patients with cervical cancer in FIGO Stage I-II. The surgical procedure consisted of systematic pelvic lymphadenectomy with removal of the common/external/internal (obturator) iliac lymph node chains, followed by radical hysterectomy depending on the clinical stage, or by Piver's type II radical laparohysterectomy for Stage IA2 carcinoma and Piver's type-III laparohysterectomy for Stage IB or Stage II carcinoma. After removal by a technique not yet described in the literature, the lymph nodes were processed directly in the operating room. HPV DNA testing was done using a cytobrush device. At the end of this operation, the lymph nodes were sent to the hospital's pathologist for metastasis detection. *Results:* The correlation between a positive HPV DNA test in the cervix and lymph node metastasis was non significant ($p < 0.63$). By contrast, the correlation between a positive HPV DNA test in the lymph nodes and lymph node metastasis was highly significant ($p < 0.005$), as was the correlation between positive HPV DNA tests in the cervix and lymph nodes ($p < 0.005$). Finally, the correlation between disease stage and positive HPV DNA testing in the lymph nodes was also significant ($p < 0.05$). *Conclusions:* In conclusion, the technique that we used for HPV DNA extraction appears safe and reproducible. The results are comparable with, if not better, than those obtained with other techniques reported in the literature. The presence of HPV DNA in the lymph nodes is probably an early indicator of metastasis and as such it could be used as a predictor of relapse. Normally untreated patients who have this marker could then receive adjuvant therapy.

Key words: HPV DNA detection; Cervical cancer; New technique.

Introduction

Carcinoma of the cervix is the second most common malignancy in women, with about 500,000 estimated new cases in 2007, 80% of them in developing countries [1]. There are substantial geographic disparities in the incidence of cervical carcinoma, mostly due to differences in the spread of screening programs for cancer prevention in the different areas of the world.

Cervical carcinoma is the first cancer that the World Health Organization (WHO) has recognized as entirely attributable to infection [2]. It is caused by a genital human papilloma virus (HPV) infection [2]. Mean age at onset of cervical carcinoma is about 52 years. Case distribution shows a bimodal pattern, with a peak around 35-39 years and another peak between 60 and 64 years [2]. The survival rate at two years from diagnosis is about 72%. The mortality rate is 1,100 cases per year.

HPV belongs to the family of viruses known as *Papovaviridae*. It is encased in a non-enveloped icosahedral capsid composed of 72 pentavalent capsomeres and has a diameter of about 55 nm [3, 4]. The HPV genome contains a double-stranded, circular supercoiled DNA molecule with about 8,000 base pairs and is associated with histonic proteins derived from the cells where viral replication occurred [3, 4]. The E6 and E7 proteins coded for by the HPV genome bind to the p53 and pRB oncosuppressors, respectively, and degrade their function. This binding capacity is greatly enhanced for high-risk HPV types (HPV-HR) [3, 4].

Today, mounting evidence suggests that this process of degradation and deregulation is not triggered by the virus, but it already exists due to other causes (environmental, chemical, etc.). The presence and integration of HPV-HR in cells with damaged DNA may prevent damage repair and favor change into a malignant phenotype.

HPV-HR is currently considered necessary but not sufficient to explain the evolution of the natural history of cervical carcinoma. Alongside the presence of HPV-HR, other important factors are: persistence of infection, integration into the cell genome, and the viral load.

Other cellular targets involved in carcinogenesis are reported in Tables 1 and 2.

Materials and Methods

The study was conducted prospectively at the University of Parma Obstetrics and Gynecology Clinic in the years 2006-2008 and at the University of Padua Obstetrics and Gynecology Clinic in the years 2004-2006.

Inclusion criteria were: histologic diagnosis of cervical squamous carcinoma or cervical adenocarcinoma, Stage I or Stage II cancer, no previous adjuvant chemotherapy or radiation therapy, and American Society of Anesthesiologists (ASA) class not exceeding III. In the overall period from November 2004 to September 2008, 18 patients were eligible for the study.

In each patient, clinical/instrumental staging was done by routine blood tests, pelvic magnetic resonance imaging (MRI),

Revised manuscript accepted for publication June 12, 2009

Table 1. — *Target molecules of the E6 viral protein.*

Target molecules of E6	Involved biological action
E6AP/p53	Degrades p53 and inhibits apoptosis
PDZ-domain-containing proteins	Degrades PDZ proteins resulting in loss of cell polarity
CAL	Inhibits cellular vesicle transport
NFX1-91	Degrades NFX1-91, activates hTERT, immortalizes cells
Paxillin	Interferes with the interaction between paxillin and focal adhesion kinase
IRF3	Inhibits IRF3 transcriptional activity, inhibits INF signal induction
Bak	Degrades Bak and inhibits apoptosis
FADD	Degrades FADD and inhibits apoptosis
Procaspase-8	Degrades procaspase-8 and inhibits apoptosis
GADD 34/PP1	Suppresses apoptosis
Tyk2	Modifies Tyk2 activation by altering the IFN signal
CBP/p300	Down-regulates p53 activity via a transcriptional coactivator
MCM7	Induces chromosomal abnormalities
T2C2	Activates the mTOR signal
BRCA1	Inhibits the ER signal

Table 2. — *Target molecules of the E7 viral protein.*

Target molecules of E7	Involved biological action
pRb	Modifies the E2F-pRb complex (E2F-mediated transcription)
Cyclin A	Regulates the cell cycle (via pRb binding)
Cyclin E	Regulates the cell cycle (via p107 binding)
p27	Binds to and inactivates CDK inhibitor p27
p21	Binds to and inactivates CDK inhibitor p21
AP1	Interacts with and transactivates the AP1-family transcriptional factors
TBP	Deregulates TBP-mediated transcription
S4 subunit of the 26 S proteosome	Targets pRb degradation
MPP2	Activates transcriptional activator MPP2
hTid 1	Replicates the genome
p48	Down-regulates the alpha-mediated INF signal
M2 pyruvate kinase	Modulates M2 pyruvate kinase activity
p600	Favors cell transformation (by modifying cell anchorage)
Mi2	Produces an HDAC complex favoring E2F2-mediated transcription
IRF1	Modifies IRF1 activity

abdominopelvic computed tomography (CT)-scan, chest X-ray, Pap smear, colposcopy with targeted biopsy, and HPV DNA typing in the cervix preoperatively and in lymph nodes intraoperatively prior to formalin preservation.

The surgical procedure consisted of systematic pelvic lymphadenectomy with removal of the common/external/internal (obturator) iliac lymph node chains, followed by radical hysterectomy depending on the clinical stage, or by Piver's type-II radical laparohysterectomy for Stage IA2 carcinoma and Piver's type-III laparohysterectomy for Stage IB or Stage II carcinoma.

After removal by a technique not previously described in the literature, the lymph nodes were processed directly in the operating room. HPV DNA testing was done using a cytobrush device. At the end of this operation, the lymph nodes were sent to the hospital's pathologist for metastasis detection.

HPV DNA typing was performed using the INNO-LiPA HPV Genotyping Extra assay (Innogenetics NV, Ghent, Belgium). With this method, part of the L1 region of the HPV genome is amplified using the SPF10 primer set and the resulting biotinylated amplicons are then denatured and hybridized with specific oligonucleotide probes. An additional set of primers for the amplification of the human HLA-DPB1 gene is included to monitor sample quality and extraction. After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase is added, which binds to any biotinylated hybrid previously formed. Incubation with the BCIP/NBT chromogen yields a purple precipitate and the results can be visually interpreted. An amplification kit is available for standardized preparation of biotinylated amplified material. This amplification kit is based on the polymerase chain reaction (PCR) and uses the SPF10 primers. Amplification products are subsequently hybridized using a single typing strip on which 28 sequence-specific DNA probe lines and four control lines are fixed.

In compliance with the International Federation of Gynecology and Obstetrics (FIGO) guidelines, patients with HPV-positive lymph nodes or non-disease-free surgical borders were given adjuvant radiation therapy. Subsequently, the patients were scheduled for a clinical/instrumental follow-up visit at four-month intervals.

Statistical analysis was done using the SPSS 15 software. The tests used were Spearman's rho to correlate the presence of HPV DNA in lymph nodes with lymph node metastasis, and chi-square to correlate HPV status, lymph nodes metastasis and disease relapse; p values were considered statistically significant at $p < 0.05$.

Results

A total of 18 patients were recruited for the study. Their clinical characteristics are reported in Table 3.

Mean age was 55 years (confidence interval [CI]: 36-69). Only one patient had a previous history of surgery for stomach cancer. Fifteen patients had taken estrogen/progestin drugs in the past for an average of six months. Only three patients were nulliparous.

Table 3. — *Preoperative characteristics of the study patients.*

Preoperative characteristics	Total (n = 18)
Mean age	55
FIGO staging	
- IA	4
- IB	6
- IIA	5
- IIB	3
Tumor grading	
- G1	0
- G2	10
- G3	8
Histologic type	
- Squamous carcinoma	15
- Adenocarcinoma	3
Size of primary lesion	
- < 4 cm	18
- > 4 cm	0
HPV strain in the cervix	
- HPV-16	12
- HPV-18	1
- HPV-31	1
- HPV-33	3

All patients had Pap smear evidence of H-SIL. The colposcopic results were dense acetowhite epithelium in 12 patients (67%), coarse mosaicism in three (25%), punctation in one, and a transformation zone with clear-cut borders in one.

Ectocervical biopsy performed in 15 patients (83%) detected squamous carcinoma in all cases. The remaining three patients had cervical adenocarcinoma. Ten patients (56%) had a scarcely differentiated tumor (G3) and eight a moderately differentiated tumor (G2). There was no G1 tumors in our case series.

HPV DNA testing performed in conjunction with colposcopy was positive in 17 cases (Table 3). HPV DNA typing detected the HPV-16 strain in 12 patients (71%) and the -33 strain in three (18%). One patient (6%) tested positive for the -31 strain, one for both the -31 and the -54 strain, and one for the -18 strain.

On clinical staging, all tumors were 4 cm or less in diameter.

The chosen surgical procedure was Piver's type-II radical hysterectomy in four patients with Stage IA2 cancer, and Piver's type-III radical hysterectomy in 14 patients, including six with Stage IB1 cancer, five with Stage IIA, and three with Stage IIB. Systematic pelvic lymphadenectomy was performed in all patients. Adnexa were preserved in only one 36-year-old woman. A total of 13 lymph nodes were removed on average (CI, 3-20).

HPV DNA in the lymph nodes was detected in 15 patients (83%), including 12 (80%) with the HPV-16 strain and three (20%) with the -33 strain (Table 4). The HPV strain correspondence rate between the cervix and lymph nodes was 83% for the -16 strain and 66% for the -33 strain.

Table 4. — *Preoperative characteristics of the study patients.*

Postoperative characteristics	Total (n = 18)
FIGO staging	
- IA2	4
- IB1	6
- IIA	5
- IIB	3
Tumor grading	
- G1	0
- G2	12
- G3	6
Lymph node metastasis	8
Total number of removed lymph nodes	175
Average number of removed lymph nodes (range, 3-30)	13
Piver's radical hysterectomy	
- Type II	4
- Type III	14
Adjuvant radiation therapy	8
- IA2	1
- IB1	0
- IIA	4
- IIB	3
HPV strain in lymph nodes	
- HPV-16	12
- HPV-18	0
- HPV-31	0
- HPV-33	3

Table 5. — *Lymph node metastasis.*

Characteristics	Positive HPV DNA testing in the cervix	Positive HPV DNA testing in lymph nodes	Lymph node metastasis	Total
FIGO staging				
- IA	4	2	1	4
- IB	5	4	0	6
- IIA	5	5	4	5
- IIB	3	3	3	3
Histologic type				
- Squamous carcinoma	14	12		
- Adenocarcinoma	3	3		
Lymph node metastasis	8	7	–	8

Table 6. — *Correlation between HPV DNA in the cervix and HPV DNA in the lymph nodes.*

Stage	G2	G3	CC-	CC+	LNF+	LNF-	M	Total
IA	4	0	0	4 (16)	2 (16)	2	1ç	4
IB	3	3	1	2 (16); 1 (18); 1 (31); 1 (33)	2 (16); 3 (33*)	1§	0	6
IIA	2	3	0	3 (16); 2 (33)	5 (16)	0	4	5
IIB	0	3	0	3 (16)	3 (16)	0	3	3

Abbreviations and cross-references:
CC-/CC+: Negative/positive HPV DNA testing in the cervix.
LNF-/LNF+: Negative/positive HPV DNA testing in lymph nodes.
M: Lymph node metastasis.
* 1 LNF+ patient (HPV-33) tested negative for HPV DNA in the cervix.
§ 1 LNF- patient tested positive for HPV DNA (HPV-31) in the cervix.
ç 1 patient with lympho-vascular space infiltration tested negative for HPV DNA in lymph nodes.
Isolated HPV strains are shown in parentheses.

Table 7. — *Correlation between HPV DNA in lymph nodes and HPV DNA in the cervix.*

Characteristics	Absence of metastasis, negative HPV DNA testing	Absence of metastasis, positive HPV DNA testing	Presence of metastasis, positive HPV DNA testing	Total
FIGO staging				
- IA	2	2	0	4
- IB	1	5	0	6
- IIA	0	1	4	5
- IIB	0	0	3	3
HPV strain in primary lesion				
- HPV-16	1	5	6	12
- HPV-18	0	1	0	1
- HPV-31	1	0	0	1
- HPV-33	0	1	2	3

Final histologic diagnosis confirmed the presence of squamous cell carcinoma and adenocarcinoma in 100% of patients. Final grading was overestimated in one case (there were actually 12 G3s) and underestimated in another one (six G2s).

Eight of the 18 patients (44%) had lymph node metastasis with lymph-vascular space invasion. Of these patients with metastasis, one (13%) had Stage IA cancer, four (50%) Stage IIA, and three (37%) Stage IIB (Table 5).

Among the eight patients with lymph node metastasis, six (75%) showed viral strain correspondence between the cervix and lymph nodes (HPV-16) (Table 6).

One patient with lymph node metastasis tested negative for HPV DNA in the lymph nodes. Conversely, HPV DNA testing was positive in eight patients with no histologic evidence of the disease in the lymph nodes (Table 7).

Eight patients were given pelvic radiation therapy (Table 4). The average follow-up period was 25 months (range, 1-48). Currently, none of these patients is showing pelvic or distant relapse.

The correlation between a positive HPV DNA test in the cervix and lymph node metastasis was non significant. By contrast, the correlation between a positive HPV DNA test in lymph nodes and lymph node metastasis was highly significant ($p < 0.005$), as was the correlation between positive HPV DNA tests in the cervix and in the lymph nodes ($p < 0.005$). Finally, the correlation between disease stage and positive HPV DNA testing in the lymph nodes was also significant ($p < 0.05$).

Using Spearman's test to compare positive HPV DNA testing in the cervix and lymph nodes and lymph node metastasis with respect to disease stage, statistical significance was reached only for the correlation between disease stage and lymph node metastasis ($p < 0.05$).

Conclusions

Cervical carcinoma is still a major health concern: worldwide, it is the second most common malignancy in women, with about 500,000 estimated new cases in 2007, 80% of which occurred in developing countries [1].

Basically, the management of cervical carcinoma requires surgical resection of the primary lesion and its spread pathways. In the initial stages of the disease (I and II), surgery is the treatment of choice when there are no absolute contraindications to it. Radiation therapy should be performed only in more advanced stages and as adjuvant therapy.

Survival is closely related to disease stage. Lymph node involvement is one of the major predictors of a poor prognosis [5]. The presence of the viral genome in metastatic lymph nodes was described in 1986 by Lancaster *et al.* [6]. The role of HPV DNA in the lymph nodes is still being studied. However, the presence of HPV DNA does appear to be a precursor of microscopically visible metastases. After infiltrating the lymph-vascular space and colonizing lymph nodes, the virus may eventually produce micrometastases and then macroscopically visible metastases.

Our study showed that the HPV DNA isolated in the cervix was mostly the HPV-16 strain, which is also the most common strain described in the literature. The other isolated strains (HPV-18, -31, and -33), were also among those associated with the highest risk of malignancy. The HPV DNA isolated in the lymph nodes was again the HPV-16 strain in most cases and the -33 strain in a very small number of other cases.

In our study, the search for the HPV genome was done in fresh samples; by contrast, all other studies reported in the literature had used either frozen or paraffin tissue sections. Fourteen of our 18 patients (78%) tested positive for HPV DNA both in the cervix and lymph nodes and this finding was statistically significant ($p < 0.005$).

In 61% of our cases, the HPV strain isolated in the cervix was the same found also in the lymph nodes. The detection of different strains in the cervix and lymph nodes could be explained by the fact that initially HPV infection is often caused by multiple strains. In addition, commercially available tests only enable detection of the virus with the highest viral load.

As regards the correlation between lymph node metastasis and the presence of HPV DNA in the cervix or lymph nodes, all of our eight patients with lymph node metastasis tested positive for HPV DNA in the cervix and seven also did for HPV DNA in the lymph nodes. This finding was statistically significant ($p < 0.005$).

In eight cases, we found HPV DNA in the lymph nodes even though no metastasis was present. In three cases, the patients neither tested positive for HPV DNA nor had any metastasis (Table 7).

The correlation between HPV DNA in the cervix and lymph node metastasis was not statistically significant. By contrast, the correlation between disease stage and lymph node metastasis was highly significant, as is also reported in the literature [7].

In a prospective study on 79 patients, Lukaszuk *et al.* [8] demonstrated that the concomitant presence of HPV DNA in lymph nodes and lymph node metastasis was twice the normal incidence rate. According to these authors, the presence of HPV DNA in lymph nodes is a prognostic indicator independent of disease stage. This study also indicated that the incidence of lymph node testing positive for HPV DNA is correlated both to the size of the primary lesion and invasion of the uterine body and the vagina, but is not correlated with infiltration of the parametrium and the paracolpia. Cavuslu *et al.* [9] reported the same findings in a larger case series.

Kobayashi *et al.* [10] confirmed the possibility of screening lymph nodes for the presence of HPV DNA using PCR as an early marker of lymph node metastasis and possible disease relapse. In his study of ten patients out of 236 with lymph node metastasis, HPV DNA was always present with the same genome.

Similar results were obtained by Sápy *et al.* [11], who in their case series found that the presence of HPV DNA in the cervix and lymph nodes was a reliable indicator of lymph node metastasis and disease relapse.

On the other hand, Fule *et al.* [12] reject the correlation between the presence of HPV DNA in lymph nodes and survival. In our opinion, though, their study has technical limitations, which are probably due to the viral DNA extraction technique used by the authors. DNA extraction by PCR on paraffin tissue sections rather than frozen sections or fresh samples may offer fewer guarantees of success.

All these studies indicate that HPV infection activates cell-mediated immunity. The hypothesis can then be advanced that HPV DNA molecules reach lymph nodes via the migration of metastasizing tumor cells from the primary lesion. According to some authors, necrotic tumor cells might carry viral DNA molecules to the lymph nodes through scavenger lymphocytes, which are normally responsible for the elimination of these necrotic tumor cells. Others assume a hematogenous spread,

which could explain the higher incidence of distant relapse in HPV-positive patients [13].

In conclusion, the technique that we used for HPV DNA extraction appears safe and reproducible. The results are comparable with, if not better, than those obtained with other techniques reported in the literature. The presence of HPV DNA in the lymph nodes is probably an early indicator of metastasis and as such it could be used as a predictor of relapse. Normally untreated patients who have this marker could then receive adjuvant therapy.

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