JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2003, p. 608–612 Vol. 41, No. 2
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.2.608–612.2003

Human Papillomavirus Type 16 Status in Cervical Carcinoma Cell DNA Assayed by Multiplex PCR

Krzysztof Lukaszuk,[1,2]* Joanna Liss,[1,2] Izabela Wozniak,[1] Janusz Emerich,[3] and Czesław Wójcikowski[1]

Department of Gynecological Endocrinology[1] and Department of Gynecology,[3] Institute of Obstetrics and Gynecology, Medical University, and INVICTA–Laboratory of Molecular Biology, Prophylactic Center,[2] Gdansk, Poland

Received 26 June 2002/Returned for modification 27 September 2002/Accepted 21 November 2002

Integration of human papillomavirus (HPV) DNA into host genome occurs early in cancer development and is probably an important event in malignant transformation of cervical cancer. The HPV genome integration usually disrupts *E2* gene open reading frames. It results in the lack of *E2* gene suppressor of the synthesis of E6 and E7 products which, in turn, leads to the overexpression of *E6* and *E7* genes. The oncogenic HPV types (HPV16, -18, -45, and -58) can be present as episomes or may integrate into human chromosomes. Sixty-six cervical cancer patients positive for HPV16 were tested for the presence of *E6*, *E2*, *E1*, and *L1* genes. Multiplex PCR was carried out in all cases. Using cluster analysis, the calculated ratios of *E1*/*E6*, *E2*/*E6*, *L1*/*E6*, *E1*/*E2*, and *E2*/(*E1*E6*) gene amplification products were divided into two or three statistically different groups. These were used for statistical analysis of the prevalence of specific gene types in histological types of cancer, different levels of clinical staging, and histologically confirmed nodal metastases. The statistical analysis proved a significant correlation in the ratios of *E2*/*E6* and *E1*/*E2* only. The *E2*/*E6* and *E1*/*E2* were higher in carcinoma in situ than in advanced squamous cancers. The *E2*/*E6* ratios were lower in higher clinical stages. The multiplex PCR estimation of the *E2*/*E6* ratio could be a simple method for selecting patients with a high risk of a poor outcome in a standard stage-dependent treatment procedure.**

Human papillomaviruses (HPV) are small (~8 kb) DNA viruses that cause warts and proliferative lesions in epidermal tissues. Infection of the anogenital tract by these viruses is associated with several premalignant and malignant lesions, especially dysplasia and carcinoma of the uterine cervix (18). HPV infection is the most frequent sexually transmitted disease worldwide, and up to 60% of sexually active women will become infected by HPV in the genital tract (19). Even in a healthy population, the incidence of HPV infection varies from ca. 20% in Brazil to ca. 3% in Belgium and Poland (17, 25). About 40 types of HPV with affinity to anogenital epithelium are known so far. HPV types 16, 18, 31, and 33 are considered the most important in the process of cervical cancerogenesis (12, 16). Genomic integration of an HPV DNA correlates with increased viral gene expression and cellular growth advantage (8). Integrated forms of HPV genomes are found much more frequently in cervical cancers than in cervical intraepithelial neoplasia cells (9, 14). These observations are consistent with the hypothesis that integration provides a selective advantage to cervical epithelial precursors of cervical carcinoma (8). During a common infection and in most premalignant lesions, HPV is in an episomal state (5). However, most cervical carcinomas and the cell lines derived from them maintain the HPV genome in an integrated form or in both integrated and episomal forms (3, 6). The genome integration of HPV usually disrupts *E2* gene open reading frames (6, 9). It results in the lack of *E2* gene suppression of the *E6* and *E7* products synthesis which, in turn, leads to overexpression of the *E6* and *E7* genes (1, 21, 23).

The *E2* gene product plays various roles in HPV replication, being involved in *E6* expression regulation and by indirectly controlling E1. The major role of the E2 protein in replication is to target E1, which is a weak DNA binding protein, to the *ori* (26). The replication of papillomaviruses requires both viral E1 and E2 proteins (26). The E2 protein positively regulates a transient replication of HPV and also represses the viral promoters (7). The replication rate of HPV changes reciprocally, depending on the level of E2. Thus, the *E1*/*E2* ratio is probably significant in this replication regulation (26).

Direct interaction between E2 and E1 can lower the concentration of E2 protein in the cell (24). It could, in addition to disrupting the *E2* region of an integrated HPV genome, lower its regulatory influence on *E6* and *E7* expression.

Thus, we suggest here that integration of the HPV into host genome influences the histological status of the HPV-related disease, i.e., advancement of the cervical cancer, by decreasing E2-related viral gene suppression. In order to examine this hypothesis, we attempted to indirectly measure the level of this integration by assessing the ratios of *E2* to both *E1* and *E6* gene products and to correlate the obtained ratios with clinical staging of the disease.

MATERIALS AND METHODS

Tissue samples and processing. Sixty-six cervical cancer patients treated at the II[nd] Department of Obstetrics and Gynecology of the Medical University of Gdansk from 1997 to 1998 were included in the present study. This group was selected from 107 patients suffering from cervical carcinoma. These 66 patients were previously shown to be HPV16 positive after pU-1 M/pU-2R and/or MY09/

* Corresponding author. Mailing address: Department of Gynecological Endocrinology, Institute of Obstetrics and Gynecology, Medical University of Gdansk, Kliniczna 1A, 80-402, Gdansk, Poland. Phone: 48-58-3493453. Fax: 48-58-7631476. E-mail: luka@amg.gda.pl.

TABLE 1. Primers for multiplex PCR amplification of *E6*, *E2*, *E1*, and *L1* HPV16 genes

HPV16 gene	Primer	Sequence	Genome localization (bp)	Length of PCR product (bp)
E6	PPH16A	5′-GACCCAGAAAGTTACCACAG-3′	126	268
	PPH16B	5′-CACAACGGTTTGTTGTATTG-3′	374	
E2	E2A	5′-TGCACCAACAGGATGTATAA-3′	3,066	147
	E2B	5′-TCAACTTGACCCTCTACCAC-3′	3,193	
E1	E1A	5′-ATGTTACAGGTAGAAGGGCG-3′	1,293	189
	E1B	5′-TGCTGCCTTTGCATTACTAG-3′	1,462	
L1	L1-16A	5′-TAGGTGTGGGCATTAGTG-3′	5,974	505
	L1-16B	5′-CCAGAGCCTTTAATGTATAAATCGT-3′	6,454	

MY11 PCR detection of cervical scrapes (15). Tissue samples from primary lesions were obtained during the surgical procedure. They were routinely frozen in liquid nitrogen and stored until PCR analysis. About 25 ± 5.5 mg of frozen tissue were taken for a single DNA isolation. The samples were incubated in 170 μl of proteinase K buffer (10 mM Tris, 1% sodium dodecyl sulfate, 400 μg of proteinase K) at 50°C overnight. DNA was extracted by using silica-based chromatography mini-columns (Genomic DNA PrepPlus; A&A Biotechnology, Gdansk, Poland) according to the manufacturer's recommendations and then stored at −30°C.

Before multiplex PCR was performed, we estimated the exact amounts of the isolated DNA for optimal HPV gene ratio estimation by a spectrophotometric method (Gene Quant; Pharmacia, Uppsala, Sweden). We have observed a dependence of the amounts of PCR products of different HPV genes on the number of copies of viral DNA contained in the DNA isolate used for PCR. A previously prepared internal standard for *E6* gene was used to determine the HPV DNA copy number. Finally, various amounts of the template DNA, equivalent to 40 to 210 HPV copies, were submitted to the multiplex PCR procedure.

Multiplex PCR method. PCR was performed for a simultaneous amplification of four HPV16 genes: *E1*, *E2*, *E6*, and *L1*. We decided to include the detection of *L1* gene fragment since PCR methods based on the fact that *L1* fragment detection are generally used for confirmation of HPV infection (10).

The oligonucleotide primers were designed by using Vector NTI 3.1. software (Table 1). Use of the E1A/E1B primer pair allowed the amplification of the 1,293- to 1,463-bp HPV16 *E1* gene fragment, whereas the E2A/E2B primers allowed the amplification of the 3,066- to 3,194-bp HPV16 *E2* fragment. These two products flanked a fragment which corresponds to the region between nucleotides 1417 and 2881, which is the one most frequently deleted regions during the HPV DNA integration in the host cells (2).

PCRs were carried out in 25 μl, in a reaction mixture containing 10 mM Tris-HCl (pH 8.81), 5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 μM concentrations of each of the deoxynucleoside triphosphates, 10 μM concentrations of each of the 8 primers, 0.5 μl of DyNAzyme II DNA polymerase (2 U/μl; FinnZyme), and 60 ng of template DNA (5 μl). A 2-min denaturation step at 94°C was followed by 30 cycles of amplification with a PCR thermocycler (TRIO-BIOMETRA). Each cycle included a denaturation step of 94°C for 1 min, a primer-annealing step of 55°C for 2 min, and a chain elongation step of 72°C for 2 min. The final elongation step was prolonged by 5 min to ensure a complete elongation of the amplified DNA. During the detection stage, 10-μl samples of PCR products were electrophoresed in a 10% polyacrylamide gel, silver stained, incubated for 6 min in 2% nitric acid, and then kept for 30 min in a 1-mg/ml $AgNO_3$ solution. The bands were visualized by incubating the gel in 3% Na_2CO_3. Transilluminated gel images were digitalized and analyzed by Gel Doc 2000 documentation system (Bio-Rad).

Statistical analysis. The values corresponding to the intensity and the density of the gel bands of different PCR products were obtained with the help of Quantity One 4.0 software (Bio-Rad). For the statistical evaluation and comparison of these data, we used the STATISTICA for Windows versions 5.1 and 6.0 (StatSoft, Inc., Tulsa, Okla.). From these values we calculated the ratios of *E1*, *E2*, and *L1* PCR products to *E6*. To find groups of patients differing in these ratios, we performed a cluster analysis applying the K-mean grouping method, for two, three, and four assumed clusters (K values of 2, 3, or 4, respectively).

In a case when a gene product was absent from a sample, a value of 0.0 was assigned to the respective ratio. It was earlier established that within the group under study the patients who showed a lack of some gene products (gene ratio = 0) did not differ in their clinical status from those with low ratios of these same genes.

We present here only these results of cluster analysis that were of the highest statistical significance, as assessed by analysis of variance, that is intrinsic to the clustering method (a value of F above the critical value of F for respective degrees of freedom, $P < 0.05$).

Optimization of multiplex PCR. Optimization of the multiplex PCR conditions was performed on DNA samples isolated from six cervical smears from patients initially positive for HPV16 who lacked integrated viral DNA (as assessed by molecular diagnosis) in a 6-month follow-up. Of 12 pairs of primers (for *E1*, *E2*, *E6*, and *L1*), we chose four pairs that gave strong PCR products when combined in one reaction tube. We carried out separate PCRs with each of the four pairs of primers and then a reaction containing all four primer pairs in the same reaction tube in order to further optimize the conditions for PCR. Conditions for this multiplex PCR were the same as those previously described.

HPV gene ratios in clinical samples. The optimized method was applied to clinical samples. As mentioned above, we used samples from the primary lesions of 66 patients suffering from cervical cancer. The mean age of the patients was 47 ± 10.0 years; ages ranged from 30 to 79 years. In a first step, the PCRs for *E1*, *E2*, *E6*, and *L1* HPV16 gene fragments were performed separately. All samples were then tested by a multiplex PCR single-tube reaction. Results from the clinical samples were compared with the results from multiplex PCR from HPV16 DNA from asymptomatic patients.

The ratios of *E1*/*E6*, *E2*/*E6*, *L1*/*E6*, and *E1*/*E2* gene PCR products were calculated and divided into two statistically different groups by cluster analysis as described above. In the same way, the ratio values of *E2*/(*E1***E6*) gene products were divided into three statistically different groups. These were used for statistical analysis of the prevalence of specific gene types in specific histological types of cancer, different types of clinical staging, and histologically confirmed nodal metastases.

The histological cancer types were divided into the following four categories: carcinoma in situ, advanced squamous cell keratinizing carcinoma, squamous cell nonkeratinizing carcinoma, and adenosquamous carcinomas.

Patients were in either the 0, Ia, Ib, IIa, IIb, or IIIb clinical (FIGO) stages. Due to the small number of cases, we analyzed the staging as divided into three groups as follows: (i) 0, (ii) Ia + Ib, and (iii) II (a + b) and III (a + b) (Table 2).

RESULTS

The multiplex PCR was initially performed and optimized on DNA samples from smears obtained from asymptomatic patients with transient HPV16 infections. It was assumed that whatever HPV DNA was detected remained in an episomal form (5). The ratios of HPV gene products obtained for patients without clinical neoplasia are shown in Table 3.

We then used clinical samples from 66 HPV16 *E6*-positive cervical cancer patients. In single primer pair PCRs, we did not find *E1* in 7 cases (10.6%), we did not find *E2* in 13 cases (19.7%), and we did not find *L1* in 22 cases (33.3%). Multiplex PCR was performed for each sample, and its results coincided with the results of single primer reactions. All four HPV16

TABLE 2. Clinical and pathological characteristics of cervical cancer patients participating in the study

Stage and category	n^a	Gene ratios and P values[b]															
		L1/E6			*E1/E6*			*E2/E6*			*E1/E2*			*E2/(E1*E6)*			
		Low	High	*P*	Low	High	*P*	Low	High	*P*	Low	High	*P*	Low	Medium	High	*P*
FIGO stage																	
0	7	4	3		4	3		3	4		4	3		2	4	1	
I (IA + IB)	29 (3 + 26)	22	7	0.47	18	11	0.38	17	12	0.02	22	7	0.09	16	8	5	0.001
II (IIA + IIB + IIIB)	30 (17 + 10 + 3)	19	11		23	7		26	4		25	5		26	3	1	
Histopathology																	
Squamous cell carcinoma																	
In situ	14	8	6		8	6		6	8		9	5		5	6	3	
Keratinizing	5	3	2	0.65	4	1	0.65	4	1	0.01	3	2	0.01	4	0	1	0.005
Nonkeratinizing	46	33	13		32	14		35	11		39	7		35	9	2	
Adenosquamous cell carcinoma	1	1	0		1	0		1	0		0	1		0	0	1	
Histopathology lymph nodes																	
Nonmetastatic	37	27	10	0.34	26	11	0.68	25	12	0.67	27	10	0.34	22	10	5	0.68
Metastatic	29	18	11		19	10		21	8		24	5		19	8	2	

[a] n = numbers of cases in a given category or subcategory.
[b] Cluster analysis of investigated HPV gene ratios in different clinical and histopathological groups of patients was performed. Two significantly different clusters were obtained for each ratio except for the *E2/(E1*E6)* ratio, where three clusters were found. The values of *P* were calculated as a chi-square test probability. *P* values apply to all values within the specified category.

gene products studied were found in 37 cases (56.1%). The lack of one of the detected genes (*E1*, *E2*, or *L1*) was established in 21 cases (31.8%), and the lack of two of them was established in 3 cases (4.5%). *E6* alone was found in 5 cases (7.6%). Where possible, the PCR product ratios were calculated, and the mean values of these ratios are shown in Table 3.

The ratio data were directly used for cluster analysis. We found two statistically different data clusters (i.e., subgroups with either a "high" or a "low" value of respective ratio) in all of the examined groups (Table 3).

The results of cluster analysis [mean *L1/E6*, *E1/E6*, *E2/E6*, *E1/E2*, and *E2/*(*E1*E6*) ratios for the clustered data] are shown in Table 3. Differences between the mean ratios describing the "high" and "low" group for each pair of PCR products were statistically significant (as determined by analysis of variance, $F > 90$, $P < 0.001$).

We did not find any correlation between the histological type of cervical cancer and the *E1/E6* or *L1/E6* gene product ratios. The ratios of *E2/E6* were significantly higher in carcinoma in situ than in advanced squamous cell keratinizing carcinoma (as determined by chi-square analysis, $P = 0.01$). Also, the *E1/E2* and *E2/*(*E1*E6*) ratios were higher for carcinoma in situ than for advanced squamous cell keratinizing and nonkeratinizing carcinoma as determined by chi-square analysis ($P = 0.01$ and $P = 0.005$, respectively).

Similarly, we did not find any correlation between the clinical staging and the ratios of *E1/E6* or *L1/E6* genes. On the other hand, we have found a statistically significant correlation between the increasing cancer staging and the increase of the frequency of low *E2/E6* ratios (chi square, $P = 0.02$). Also, the *E2/*(*E1*E6*) ratios were significantly higher in the lower stages (chi square, $P = 0.01$). Although the ratios of *E1/E2* were also higher for lower clinical stages, this relation was not statistically significant (chi square, $P = 0.09$).

Finally, we did not find any correlation between the occur-

TABLE 3. Cluster analysis of HPV gene multiplex PCR ratios of asymptomatic and cervical cancer smears

Gene ratio	Asymptomatic smears		Cervical cancer whole group analysis		Cervical cancer cluster analysis[a]			
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD (low, high)	*n* (low, high)	*F*	*P*
L1/E6	1.27 ± 0.28	1.03–1.67	0.47 ± 0.48	0.00–2.00	0.22 ± 0.23, 1.03 ± 0.39	45, 21	110.22	<0.001
E1/E6	1.11 ± 0.26	0.91–1.49	0.90 ± 0.62	0.00–2.8	0.58 ± 0.36, 1.59 ± 0.47	45, 21	90.71	<0.001
E2/E6	0.8 ± 0.27	0.55–1.18	0.73 ± 0.62	0.00–2.6	0.40 ± 0.32, 1.49 ± 0.49	46, 20	113.75	<0.001
E1/E2	1.5 ± 0.21	1.29–1.8	0.81 ± 0.90	0.00–5.66	0.52 ± 0.36, 1.79 ± 1.40	51, 15	103.33	<0.001
E2/(*E1*E6*)	0.001 ± 0.0005	0.0006–0.002	0.0025 ± 0.003	0.00–0.014	0.0005 ± 0.0005, 0.0036 ± 0.001, 0.01 ± 0.002[b]	41, 18, 7[b]	307.18	<0.001

[a] n, number of cases assigned to a cluster; F, value of Fisher-Snedocor test; P, significance level of difference between the clusters.
[b] Values correspond here to: low, medium, high.

rence of histologically confirmed metastases and the ratios of any two gene products studied.

DISCUSSION

Like other forms of HPV, oncogenic HPV types can also be present as episomes or may integrate with human chromosomes. The phenomenon of integration has been regarded as a potentially important mechanism for tumor progression in the cervix (transformation of dysplasia into invasive carcinoma) (3, 4, 20). The presence of the integrated, oncogenic (*E6* or *E7*) sequences may give the infected epithelium growth advantages and may contribute to increased cell proliferation and genomic instability, leading to further genetic alterations (13). The concomitant deletions in the *E1*/*E2* regions of the virus have prognostic implications in carcinoma patients (11). On the other hand, the functions of *E2* and *E1* seem to depend on their relative ratio to *E6* (28). Thus, determination of the relative quantities of *E1* and *E2* genes (as PCR products) and their respective ratios to *E6* could estimate the integration degree of HPV. The information about the level of integration of HPV DNA with a host genome and the absence of some HPV DNA sequence(s) could thus be interesting and probably useful in the prognosis of cervical cancers progression and outcome.

Thus far, the episomal and integrated forms of HPV are detected mostly by a hybridization method (8) that is labor-intensive and relatively expensive. Moreover, results obtained by hybridization inform us only about the presence or absence of either form of the HPV genome.

This is why we prepared a simple and reliable multiplex PCR method for the parallel detection of four different HPV genes: *E1*, *E2*, *E6*, and *L1* (the latter is included as a known marker of HPV infection). We believe that the use of appropriately optimized multiplex PCR for simultaneous estimation of genomic load with four HPV genes of interest has—in addition to its obvious benefits over the hybridization method—also some benefits over single PCRs for detecting one HPV gene product at a time. One of these benefits may be a higher credibility of obtained gene ratios. We have found a statistically significant difference between the *E1*/*E2* PCR product ratio for different histological types of cancer and for different stages of cancer development. The mechanism of *E1* and *E2* expression does not depend on the *E1*/*E2* ratio only. The possibility of *E1* and/or *E2* gene mutations could be one of plausible reasons. On the other hand, it is possible that even an integrated HPV genome, with a deleted *E2* sequence, could be suppressed by the product of the *E2* gene from an episomal form of HPV existing in the same cell. That is why we suggest that the ratio of *E2*/*E6* could play a role in cancerogenesis and be more diagnostically significant than simple confirmation of the HPV DNA integration with the host genome.

A different mechanism for losing of *E2*-dependent regulation was proposed by Stuenkel et al. (22). These authors suggested that the matrix attachment region-dependent HPV16 oncogene transcriptional stimulation might precede the release of *E2*-dependent repression. This matrix attachment constitutes a dominant genotype that might overcome *E2* repression, establish an enhanced *E6* and *E7* expression, and confer a phenotype favorable to carcinogenesis. There have been some studies that confirm the poor disease-free survival of cervical cancer in patients who exhibited the presence of intact *E2* (27), which could, in principle, lead to an increased ratio of *E2* to other HPV gene products. On the other hand, investigation of the whole *E2* gene sequence by PCR amplification by Vernon et al. demonstrated a lack of some parts of the *E2* gene in half of the tumors with integrated HPV16 DNA. These authors determined the disease-free survival by using the Kaplan-Meier estimate for the entire *E2* gene presence or absence. The disruption of *E2* was found to be associated with a significantly shortened disease-free survival. This finding is corroborated by our data; our multiplex PCR estimation of the *E2*/*E6* ratio in patients shows a significantly higher ratio in patients with the lower clinical stages. Thus, this could be a very simple method to select the patients with a high risk of poor outcome of standard stage-dependent treatment procedure.

A different aspect of the HPV gene detection is using this method to determine the presence of lymph node metastasis. HPV DNA in lymph nodes informs us about metastasis presence but on a different basis than that for the histological confirmation. Thus, the detection of HPV DNA could be used as an additional marker for patient follow-up. The HPV gene ratios found in lymph nodes differ from the ratios in the primary lesions studied here. We found a lower *E2*/*E6* ratio in lymph nodes containing HPV-positive metastases (K. Łukaszuk, J. Liss, J. Witkowski, and C. Wójcikowski, unpublished data).

The *E2* gene product plays various roles, since it is involved in *E6* expression regulation and, by indirectly controlling *E1*, in HPV replication. That is why we calculated the *E2* ratio to both *E1* and *E6*. The obtained results are statistically highly significant in relation to the histological type ($P = 0.005$) and the clinical staging ($P = 0.01$) of cervical cancers. The E6 and E7 oncoproteins are involved in cell transformation. Their presence is necessary for cancerogenesis. We detected the *E6* gene PCR product in all of the cervical cancer samples where HPV was present. Very different HPV detection results were obtained based on the *E6* gene detection than when we used consensus primers for *L1* detection. The presence of *L1* gene PCR products was reported to be found only in 60% of the HPV *E6* gene detection system (10). We therefore included the primers for *L1* in our system. We found the presence of *L1* in only 44 cases (66.6%). We did not find any correlation between the presence of the *L1* gene product and any of the clinical parameters. Thus, the *L1* primers could be excluded from this diagnostic system without a loss in sensitivity.

Our diagnostic system did not allow us to unambiguously differentiate the physical status of HPV16 in the cervical cancer cells. However, we suggest that the different HPV gene product ratios proposed here could be more clinically significant than the differentiation of these cancers, depending on the episomal and integrated HPV status.

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