Assessment of human papilloma viral load of archival cervical intraepithelial neoplasia by real-time polymerase chain reaction in a Turkish population

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Summary

The purpose of this study was to determine the existence, and viral load of human papilloma virus (HPV) subtypes 16 and 18 in paraffinized cervical intraepithelial neoplasia (CIN) samples by real-time polymerase chain reaction (RT-PCR). Overall 94 women were included. Of these patients 47 (50%) had CIN I, 27 (28.8%) had CIN II, and 20 (21.2%) had CIN III. HPV positivity for these three groups were 4.2%, 14.8% and 45%, respectively. HPV positivity in CIN III patients was significantly higher than CIN I (OR = 18.41, 95% CI 3.00-145.73; $p < 0.001$), and CIN II patients (OR = 4.70, 95% CI 1.00-23.76; $p = 0.05$). The difference between CIN I and II was not significant ($p = 0.18$). Viral loads were 10^2, and 10^4 copy/ml for two CIN I patients; 10^2, 10^3, and 10^5 for three CIN II patients; and 10^2, 10^3, 10^4, 10^4, 10^5, 10^5, and 10^6 copy/ml for eight patients with CIN III. Viral load of the remaining one patient could not be assessed. No significant variance was noted among the groups with respect to viral load ($p = 0.73$). RT-PCR had important advantages of detecting, typing, and quantifying at the same time. Although HPV positivity was increased significantly by the degree of lesions, this relation was not observed for viral load.

Key words: Human papilloma virus; Real-time polymerase chain reaction; Viral load; Cervical intraepithelial neoplasia.

Introduction

Although the incidence of cervical carcinoma has been decreasing in developed countries, it is still a big problem for rest of the world. Cytologic screening programs to detect cervical intraepithelial neoplasia (CIN) have led to reductions in morbidity and mortality from cervical cancer [1]. Meta-analyses have shown that the sensitivity of a cytology smear is low (30-87%) in contrast to its specificity of 86 to 100% for high-grade (CIN II or III) cervical lesions [2]. Human papilloma virus (HPV) infection is frequently detected in CIN and invasive cervical carcinoma [3]. It is estimated that > 90% of cervical squamous cell carcinomas, and > 50% of adenocarcinomas contain HPV DNA (4). Certain subtypes of HPV, such as HPV-16 and HPV-18 are most commonly associated with cervical carcinoma. Oncogenic HPVs carry the transforming E6 and E7 genes. These genes are necessary and sufficient for malignant transformation and immortalization of cervical epithelial cells [3, 5]. The detection of specific human papillomavirus E6 and E7 oncogene transcripts may be a sensitive indicator of direct involvement of viral oncogenes in the development of cervical neoplasia and carcinoma [6].

All patients with HPV infection do not develop cervical dysplasia. Viral load has been demonstrated to be an important factor to estimate who will have cervical intraepithelial neolasia [7-9]. Also some authors reported that viral load increases with the grade of CIN [9-12]. Viral load assessments have been performed by using many different techniques before the popularization of real-time polymerase chain reaction (RT-PCR), which has an additional advantages of detection, typing, and quantitation at the same time. Besides the fresh samples, also paraffin-embedded specimens have been analyzed [13]. The goal of this study was to evaluate the existence and viral loads of HPV 16 and 18 in paraffinized CIN tissue, and to investigate the possible relation with the severity of CIN by using RT-PCR.

Materials and Methods

The patients who were treated for CIN at Gazi University Hospital were evaluated retrospectively, and paraffin-embedded biopsy specimens were reviewed by co-author pathologists using the classification system of the World Health Organization.

DNA extraction: Paraffin-embedded cervical tissue sections were deparaffinized by a xylen method in our molecular microbiology laboratory. Specimens were digested in a buffer containing 20 mg/ml proteinase K (20mM $(NH_4)_2SO_4$, 75mM Tris HCl [pH 8,8] 0.1% Tween 20) at 55°C for three hours followed by ten minutes at 95°C. DNA isolation was performed by phenol-chloroform extraction and ethanol precipitation. DNA was then suspended in sterile distilled water and stored at -86°C until amplification.

DNA amplification: DNA was amplified by using consensus

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MY 09 and MY11 (5'-CGTCCMARRGGAWACTGATC-3'), (5'-CMCAGGGWCATAAYAATGG-3') (Tib Mol-biol, Germany) primers from the L1 region. A negative control and Caski cell DNA as a positive control were used during amplification. Amplification reaction was performed in a total volume of 50 ml including 45 µl of amplification mixture and 5 µl of extracted DNA. The amplification mixture contained 100 pmol of each consensus primer, 100 µM of each dNTP (dATP, dCTP, dGTP ve dTTP) and 1 unite of Taq DNA polymerase (DNA mp ltd., Hants, UK) in a buffer of 4 mM MgCl2, 50 mM KCl2, 10mM Tris HCl (pH 9). Amplification was performed in a MJ Research thermalcycler S Vt 6 vice. The mixture was first denaturated at 94°C for 5 min. Then 35 cycles including 20 sec at 94°, 45 sec at 55° and one minute at 72°C were performed, and at the end of the last cycle 7 min at 72°C was performed. PCR products were analyzed by electrophoresis through 1.5% agarose gel, and then 450 bp bands for HPV were visualized by UV light after ethidium bromide staining.

HPV genotyping and quantification: MYO9 and MY14 primer set (5'-CGTCCMARRGGAWACTGATC-3' and 5'CATACACCTCCAGCACCTAA-3') for HPV type 16, and MY11 and WD74 primary sets (5'-CMCAGGGW-CATAAYAATGG-3' and 5'GGATGCTGCACCGGCTGA) for HPV type 18 were used for positive samples. PCR amplification was performed in a light cycler 2.0 (Roche, Germany) real-time PCR device with a 10 µl total volume containing 2 µl of PCR positive samples, 1 µl of LC DNA Syber Green Master mix, 5mM $MgCl_2$, and HPV type 16 primer mix. The same procedure was performed by adding HPV type 18 primer mix. Forty-five cycles of amplification were performed and the results were determined at 530 nm; 10^2-10^8 copy/ml standards were used for positive samples, and the quantification of the positive samples were detected according to these standards once again by using a RT-PCR device.

Statistical analyses were performed using the SPSS computer program package (version 11.5 for Windows). Frequency tables were analyzed by the chi-square test, and Fisher exact test for the significance between the categorical variables. Odd ratios (OR) and the 95% confidence intervals (CI) were calculated where appropriate by the use of accurate methods. Continuous variables between the groups were analyzed by Kruskal-Wallis variance analysis. Statistical significance was set at p = 0.05.

Results

The cohort consisted of 94 women who were subjected to conization with the diagnosis of CIN. The mean age at the time of diagnosis was 47.15 ± 8.48 years (age range, 28 to 65 years). Of these patients 47 (50%) had CIN I, 27 (28.8%) had CIN II, and 20 (21.2%) had CIN III disease.

HPV DNA was detected in 15.9% (15/94) of the women. Of the patients classified as CIN I, two had HPV type 16 (4.2%). In the CIN II group, two had HPV type 16 (7.4%), and one had HPV type 18 (3.7%). Type of HPV could not be determined by RT-PCR in one sample with CIN II (3.7%). In patients with CIN III, five had HPV type 16 (25%), and three had HPV type 18 (15%). Again, type of HPV could not be assessed for one patient (5%). In addition, the HPV DNA in one sample was missed by RT-PCR. The upword rate of HPV positivity parallel to the degree of CIN was found to be statistically significant (p = 0.006, Table 1). HPV positivity in CIN III patients was significantly higher than CIN I (OR = 18.41, 95% CI 3.00-145.73; p < 0.001), and CIN II patients (OR = 4.70, 95% CI 1.00-23.76; p = 0.05). The difference between CIN II and I did not reach a significant value (OR = 3.91, 95% CI 0.55-33.66; p = 0.18).

Table 1. — *Human papilloma virus (HPV) positivity with respect to degree of cervical intraepithelial neoplasia (CIN).*

Type of HPV	CIN I (n = 47)	CIN II (n = 27)	CIN III (n = 20)
HPV type 16	2 (4.2%)	2 (7.4%)	5 (25%)
HPV type 18	–	1 (3.7%)	3 (15%)
HPV type undetermined	–	1 (3.7%)	1 (5%)
Total HPV positivity	2 (4.2%)	4 (14.8%)	9 (45%)

p = 0.006.

Viral loads were recorded as copy/ml. During this process 10^2-10^8 copy/ml standards were used. Table 2 summarizes the results of viral load in the three patient categories. Of the patients classified as CIN I, viral loads for two patients with HPV type 16 were 10^2 and 10^4 copy/ml, respectively. In two CIN II patients with HPV type 16, viral loads were 10^2 and 10^5 copy/ml, respectively. Whereas, viral load for one patient with HPV type 18 was 10^3 copy/ml. In patients with CIN III, viral loads were 10^2, 10^3, 10^4, 10^5, and 10^5 copy/ml for five patients with HPV type 16; and 10^4 and 10^6 copy/ml for two patients with HPV type 18. For the remaining one patient with HPV type 18 viral load could not be assessed. Viral load did not vary significantly among the three groups (p = 0.73).

Table 2. — *Viral loads of patients with respect to degree of cervical intraepithelial neoplasia (CIN).*

	Viral load (copy/ml)		
Type of HPV	CIN I	CIN II	CIN III
HPV type 16	10^2, 10^4	10^2, 10^5	10^2, 10^3, 10^4, 10^5, 10^5
HPV type 18	–	10^3	10^4, 10^6, X[a]

[a]For one patient viral load could not be determined.

Discussion

Since there is a long premalignant stage of cervical cancer, detection and effective treatment of those women with preinvasive diseases significantly decrease the rate of invasive cancer. HPV is considered to be a main cause of CIN and cervical cancer [14]. By the advent of new techniques, today more than 100 different HPV types have been identified, and classified as low, intermediate or high risk according to the potential to induce malignant transformation [4, 15, 16]. Many different methods have been developed to detect virus, to quantify load, and to identify the cellular changes [17-19]. Besides the detection of existence of HPV, quantification has also gained significant popularity. The previously published series generally used conventional PCR-based techniques. Zerbini *et al.* evaluated 176 cytological specimens of different cervical lesions to show the distribution and viral load of most prevalent high-risk HPVs by PCR-ELISA [20]. In their population HPV 16 was positive in 57.6% of the samples. Only HPV 16 load was found to

be associated with the severity of cervical disease. Swan *et al.* also showed that only the amount of HPV 16 DNA differed significantly among the tested high-risk HPV types 16, 18, 31, and 45 [21]. Hernandez *et al.* analyzed 182 patients with diagnoses of CIN I-III by using the Hybrid Capture 2 (HC2) test to detect HPV DNA and light measurements for viral load [12]. A total of 79.4% of patients with CIN I, and 93.7% of CIN II-III were positive for HPV DNA, and CIN II-III had the highest association with viral load (OR = 365.8, 95% CI 94.7-1412). In addition median viral load increased significantly by CIN lesion grade ($p < 0.001$). Sun *et al.* [22] reported a median 1.42 and 11.87 relative light unit (RLU) ratio, respectively, for 19 LSIL and 32 HSIL/SCC patients with a significant variance (OR = 35, 95% CI 4.2-294.5). Abba *et al.* [23] included 73 LGSIL and 74 HGSIL samples to show viral load with a low stringency PCR method, and reported that the HGSIL group had significantly higher values of viral load than LGSIL patients (OR = 3.10, 95% CI 1.54-6.23).

Besides the series only detecting HPV positivity, some authors have questioned the further impact of previous viral load on the development of CIN. Ylitalo *et al.* evaluated HPV 16 load of 2,081 smears of 478 patients with carcinoma in situ by using quantitative PCR assay, and reported that women with a high viral load had 30 times more risk than HPV 16 negative women more than a decade before diagnosis [24]. In a large longitudinal French cohort study 781 patients with normal, ASCUS or LGSIL cytology were followed by six-month intervals for a median period of 22 months [9]. Progression to CIN II-III or cancer was only seen in patients with persistent high-risk HPV infection, and the risk was increased with initial high viral loads. Therefore, they suggested to use high viral load as a short-term marker of progression toward precancerous lesions. By the popularization of RT-PCR, similar series were also published. In their prospective study including 125 patients, Van Duin *et al.* demonstrated that in women with normal cytology, an incresed HPV 16 load quantified by RT-PCR was related to the increased risk of developing a CIN lesion [25]. Moberg *et al.* analyzed 2,747 archival Pap smears of 457 patients with carcinoma in situ to assess the viral load of ten different types of HPV [26]. Viral load was predictive of future risk of CIS, and the highest relation was reported for HPV 16 with an OR of 36.9. In a comparative study, Gravitt *et al.* reported that in the presence of multiple coinfections HC2 overestimated type-specific viral load, and it was suggested that this may be the explanation of controversial results obtained in some case-control studies [27]. In the current study HPV 16 and 18 were tested for positivity in different degrees of CIN. HPV positivity in CIN III patients was significantly higher than CIN I (OR = 18.41), and CIN II patients (OR = 4.70). The difference between CIN II and I did not reach to a significant value. Interestingly, the positivity rate especially for CIN I and II lesions were extremely lower than the value reported in the literature. Our microbiology laboratory has a lot of experience with the RT-PCR technique, therefore we thought that the usage of formalin-fixed paraffin-embedded samples precluded obtaining higher results. Some other authors have also mentioned the underestimation of HPV prevalence in paraffin-embedded samples. A detailed study comparing the RT-PCR and conventional PCR in archival cervical cancer tissue showed that these two techniques had similar detection rates [13]. Strikingly they observed an inhibitory effect of formalin fixation and paraffin embedding on the evaluation of viral load. Additionally, we did not find significant variance in viral loads of the three grades of CIN. Although it has been reported to be increased with the degree of disease as mentioned in the series discussed above, some investigators did not observe this relation. Lorincz *et al.* followed 20,810 women for ten years after measuring viral load at enrollment [28]. By HC2 testing of cervicovaginal lavages, it was reported that presence of HPV was strongly correlated with risk of CIN III but no association with viral load was observed.

In summary, this is the first study evaluating the Turkish population with regard to viral load of cervical intraepithelial neoplasia. Although HPV positivity was increased significantly by the degree of lesions, this relation was not observed for viral load. RT-PCR had important advantages of detecting, typing, and quantifying at the same time. By the publication of further series we will have more information about the advantageous and disadvantageous characteristics of this relatively new method.

References

[1] Parkin D.M., Pisani P., Ferlay J.: "Global cancer statistics". *CA Cancer J. Clin.,* 1999, *49,* 33.

[2] Nanda K., McCrory D.C., Myers E.R. *et al.*: "Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytologic abnormalities: a systematic review". *Ann. Intern. Med.,* 2000, *132,* 810.

[3] zur Hausen H.: "Papillomaviruses in anogenital cancer as a model to understand the role of viruses in human cancers". *Cancer Res.,* 1989, *49,* 4677.

[4] Bosch F.X., Manos M.M., Munoz N., Sherman M., Jansen A.M., Peto J. *et al.*: "Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group". *J. Natl Cancer Inst.,* 1995, *87,* 796.

[5] Woodworth C.D., Doniger J., DiPaolo J.A.: "Immortalization of human keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma". *J. Virol.,* 1989, *63,* 159.

[6] Wang-Johanning F., Lu D.W., Wang Y., Johnson M.R., Johanning G.L.: "Quantitation of human papillomavirus 16 E6 and E7 DNA and RNA in residual material from ThinPrep Papanicolaou tests using real-time polymerase chain reaction analysis". *Cancer,* 2002, *94,* 2199.

[7] Cuzick J., Terry G., Ho L., Hollingworth T., Anderson M.: "Human papillomavirus type 16 in cervical smears as predictor of high-grade cervical intraepithelial neoplasia". *Lancet,* 1992, *339,* 959.

[8] Schneider A., Zahm D.M., Greinke C., Kirchmayr R., Nindl I.: "Different detectability of high-risk HPV in smears from incident and prevalent high-grade squamous intraepithelial lesions of the cervix". *Gynecol. Oncol.*, 1997, *65*, 399.

[9] Dalstein V., Riethmuller D., Pretet J.L., Le Bail Carval K., Sautiere J.L., Carbillet J.P. *et al.*: "Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study". *Int. J Cancer,* 2003, *106,* 396.

[10] Healey S.M., Aronson K.J., Mao Y., Schlecht N.F., Mery L.S., Ferenczy A., Franco E.L.: "Oncogenic human papillomavirus infection and cervical lesions in aboriginal women of Nunavut, Canada". *Sex. Transm. Dis.,* 2001, *28,* 694.
[11] Sun C.A., Liu J.F., Wu D.M., Nieh S., Yu C.P., Chu T.Y: "Viral load of high-risk human papillomavirus in cervical squamous intraepithelial lesions". *Int. J. Gynaecol. Obstet.*, 2002, *76*, 41.
[12] Hernandez-Hernandez D.M., Ornelas-Bernal L., Guido-Jimenez M., Apresa-Garcia T., Alvarado-Cabrero I., Salcedo-Vargas M. *et al.*: "Association between high-risk human papillomavirus DNA load and precursor lesions of cervical cancer in Mexican women". *Gynecol. Oncol.,* 2003, *90,* 310.
[13] Biedermann K., Dandachi N., Trattner M., Vogl G., Doppelmayr H., More E. *et al.*: "Comparison of real-time PCR signal-amplified in situ hybridization and conventional PCR for detection and quantification of human papillomavirus in archival cervical cancer tissue". *J. Clin. Microbiol.*, 2004, *42*, 3758.
[14] zur Hausen H.: "Immortalization of human cells and their malignant conversion by high risk human papillomavirus genotypes". *Semin. Cancer Biol.,* 1999, *9*, 405.
[15] Schiffman M.H., Bauer H.M., Hoover R.N., Glass A.G., Cadell D.M., Rush B.B. *et al.*: "Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia". *J. Natl. Cancer Inst.*, 1993, *85*, 958.
[16] Keerti V.S., Howley P.M.: "Papillomaviruses". In: Fields B.N., Knipe D.M., Howley P.M. *et al.* (eds.). Virology. Philadelphia: Lippincott-Raven, 1996, 2, 2077.
[17] Josefsson A.M., Magnusson P.K., Gyllensten U.B.: "Human papilloma virus detection by PCR and typing by dot-blot". In: Peeling SP (ed.). Sexually transmitted diseases: methods and protocols. Humana Pres, Inc., Totowa, N.J. 1999, 171.
[18] Jacobs M.V., Walboomers J.M., van Beek J., Voorhorst F.J., Verheijen R.H., Meijer C.J. *et al.*: "A quantitative polymerase chain reaction-enzyme immunoassay for accurate measurements of human papillomavirus type 16 DNA levels in cervical scrapings". *Br. J. Cancer,* 1999, *81*, 114.
[19] Hart K.W., Williams O.M., Thelwell N., Fiander A.N., Brown T., Borysiewicz L.K., Gelder C.M.: "Novel method for detection, typing, and quantification of human papillomaviruses in clinical samples". *J. Clin. Microbiol.,* 2001, *39,* 3204.
[20] Zerbini M., Venturoli S., Cricca M., Gallinella G., De Simone P., Costa S. *et al.*: "Distribution and viral load of type specific HPVs in different cervical lesions as detected by PCR-ELISA". *J. Clin. Pathol.,* 2001, *54*, 377.
[21] Swan D.C., Tucker R.A., Tortolero-Luna G., Mitchell M.F., Wideroff L., Unger E.R. *et al.*: "Human papillomavirus (HPV) DNA copy number is dependent on grade of cervical disease and HPV type". *J. Clin. Microbiol.*, 1999, *37,* 1030.
[22] Sun C.A., Lai H.C., Chang C.C., Neih S., Yu C.P., Chu T.Y.: "The significance of human papillomavirus viral load in prediction of histologic severity and size of squamous intraepithelial lesions of uterine cervix". *Gynecol. Oncol.*, 2001, *83,* 95.
[23] Abba M.C., Mouron S.A., Gomez M.A., Dulout F.N., Golijow C.D.: "Association of human papillomavirus viral load with HPV16 and high-grade intraepithelial lesion". *Int. J. Gynecol. Cancer*, 2003, *13*, 154.
[24] Ylitalo N., Sorensen P., Josefsson A.M., Magnusson P.K., Andersen P.K., Ponten J. *et al.*: "Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study". *Lancet,* 2000, *355*, 2194.
[25] van Duin M., Snijders P.J., Schrijnemakers H.F., Voorhorst F.J., Rozendaal L., Nobbenhuis M.A. *et al.*: "Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance". *Int. J. Cancer*, 2002, *98,* 590.
[26] Moberg M., Gustavsson I., Gyllensten U.: "Real-time PCR-based system for simultaneous quantification of human papillomavirus types associated with high risk of cervical cancer". *J. Clin. Microbiol.,* 2003, *41,* 3221.
[27] Gravitt P.E., Burk R.D., Lorincz A., Herrero R., Hildesheim A., Sherman M.E. *et al.*: "A comparison between real-time polymerase chain reaction and hybrid capture 2 for human papillomavirus DNA quantitation". *Cancer Epidemiol. Biomarkers Prev.,* 2003, *12,* 477.
[28] Lorincz A.T., Castle P.E., Sherman M.E., Scott D.R., Glass A.G., Wacholder S. *et al.*: "Viral load of human papillomavirus and risk of CIN3 or cervical cancer". *Lancet*, 2002, *360*, 228.

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