DETECTION OF HPV 16 AND HPV 18 VIRAL LOADS BY REAL TIME PCR IN WOMEN WITH CERVICAL DYSPLASIA

RAMONA GABRIELA URSU [1*], MIRCEA ONOFRIESCU [2], DRAGOŞ NEMESCU[2], ROXANA NEMESCU[3], LUMINIŢA SMARANDA IANCU[1, 4]

Keywords: viral load, qPCR, HR HPV types, biomarker.

Abstract. Viral load of high risk HPV types 16 and 18 can be useful biomarkers in detecting women with cervical dysplasia in early stage, prior to development of cervical cancer. Purpose of the study was to assess the clinical utility of viral load determination for HPV 16 and 18 in relation with the severity of cervical dysplasia and the association of classical risk factors. The HPV 16 and 18 positive women were selected from a cohort study, using one HPV DNA test for screening. METHODS: Viral load quantification was performed with Real Time PCR MX3005P. RESULTS: The viral load for HPV 16 was between 3.45 - 7.177 x 10^6 copies / µl, and 1.138 x 10^3 to 7.119 x 10^4 for HPV 18. CONCLUSIONS: Viral load of high risk HPV types seems to be one sensitive and objective biological marker in detecting women at risk for developing cervical cancer. No significant association was fount for the risk factors investigated.

INTRODUCTION

Human papillomaviruses (HPV) are the cause of cervical cancer, and among them, HPV 16 and 18 are the leading types, because they alone cause over 70% of cervical cancers (Walboomers JM *et al.*, 1999). Natural history of HPV infection shows that in 80% of High Risk HPV (HR HPV) infections there are transitory infections, in 20% of cases, in 2 - 4 years will appear productive infection and after 10 – 30 years will be developed cervical cancer. To evaluate the risk of HR HPV positive women to develop cervical cancer it was proposed several biological markers: virus related markers, disease markers and cell cycle related markers (Meyer CJLM, 2009). One of the virus related factors is the viral load of HR HPV types and it is presumed that a higher risk for cervical neoplasia is associated with higher viral loads of high-risk HPV types, in particular HPV 16 (WHO & IARC, 2007). The association between viral load and cervical disease varies with the HPV type, the physical state of the virus and the heterogeneity of the cervical lesion (Woodman CB et al., 2007). A few reports stated that viral load of HR HPV (16, 18, 31 and 33) can be used to select women wich need a more aggresive treatment, because they are consider to have high grade cervical intraepithelial lesions (Cricca M *et al*., 2007, Snijders PJ *et al*., 2006). Our aim in this study was to evaluate the clinical usefulness of copies number of DNA HPV 16 and 18 in relation with the severity of cervical dysplasia and classical risk factors known as oncogenic.

MATERIAL AND METHODS

In the period between September 2009 – December 2010 we invited to participate in one HPV prospective genotype prevalence study women with abnormal Pap smear and with colposcopic suspicion of HPV infection. All the study participants have signed the informed consent approved by the Bioethical Committee of "Gr. T. Popa" University of Medicine and Pharmacy, Iasi.

From 267 genotyped samples, we identified 26 (9.7 %) positive for HPV 16 and 9 (3.4 %) positive for HPV 18, using Linear Array HPV Genotyping Test (*ROCHE®*). HPV/DNA was purified with High Pure PCR Template kit (ROCHE®). Viral load was assed with *2 x Precision TM Mastermix*, *Path-HPV16 Real-time PCR detection kit for Human Papillomavirus, Path-HPV18 Real-time PCR detection kit for Human Papillomavirus kits (PRIMER DESIGN®)* and MX3005P instrument (*STRATAGENE*).

The HPV 16 and 18 specific primers and probes mix can be detected through the FAM channel. The primers and probes mix provided exploits the TaqMan principle. During PCR amplification, forward and reverse primers hybridize to the HPV16 and 18 DNA/cDNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on real-time PCR platforms.

Preparation of standard curve dilution series was performed using the positive control in dilution from 2 x 10^6/µl up to 2 copies / µl. Pathogen detection mix contained: 10 µl 2 x Precision TM Master Mix,1 µl Pathogen Primer/Probe mix, 4 µl RNA se / DNAse free water: the final volume was 15 µl. Endogenous ACTB detection mix contained: 10 µl 2 x Precision TM Master Mix,1 µl Endogenous ACTB Primer/Probe mix , 4 µl RNA se / DNA se free water. 15 µl of each of this two mixes was pipetted into each well according to real-time PCR experimental plate set up. 5µl of diluted DNA wad added into each well, according with the experimental plate set up. Amplification Protocol supposed one cycle for enzyme

activation – 10 minutes / 95 ° C and the 50 cycles each one with one denaturation step – 10 s / 95° C and one for data collection using FAM channel - 60 s / 60 ° C.

RESULTS AND DISCUSSION

The mean age of HPV 16 positive women was 36.31 years old (limits 23 – 59). 6 (23.1%) patients had never performed one cytologic exam, 1 (3.8%) had one normal Pap smear and also 1 (3.8%) has atypical cells, 3 (11.5%) had ASCUS *(Atypical Squamous Cells of Undetermined Significance)*, LGSIL (*Low Grade Squamous Intraepithelial Lesion*), and ASCH (*Atypical Squamous Cells for which a High-grade lesion cannot be exclude*d), each, and 9 (34.6%) were detected with HGSIL (*High Grade Squamous Intraepithelial Lesion*). Regarding risk factors, 4 (15.4%) women were smokers and 9 of them (34.6%) declared that they used oral contraceptive in the past. 16 (61.5%) patients presented unique infection with HPV 16 and 10 (38.5%) were detected with multiple HPV types infections, besides HPV 16.

The mean age of HPV 18 positive patients was 30.8 (limits 21 – 41 years). Two had one normal Pap test, one was never testes by cytologic exam, and one had ASCUS and two LGSIL. One patient declared having more than 3 sexual partners, another declared 3 abortion and another patients had genital warts.

The parameters of the standard curves was calculated by the Stratagene software: efficiency = 97,3%, RSq (the linearity is denoted by the R squared (Rsq) value - R2 or Pearson Correlation Coefficient) = 0.988, slope = - 3.389 for HPV 16 and efficiency = 93.1%, RSq = 0.993, and slope = - 3.500 for HPV 18. All this parameters was between the normal limits (efficiency: 90 – 110%, RSq: 0.985 – 1.00, linearity / slope: –3.1 and –3.6). The NTC (negative controls) proved that there was no contamination of the experiments. The Ct values for ACTB control (beta actin gene) confirmed that the cervical cells were optimal collected. The amplifications plots for HPV 16 positive samples and the standard curve can be seen in figure 1 and figure 2.

The samples was tested in duplicate. The Ct / Qc (cycle threshold / quantification cycle) values were between 19.68 and 33.94. The mean of viral load of HPV 16 was 684515,68 copies (limits 3,45 - 7.177 x 10^6 copies). Ct values for HPV 18 were between 25.44 – 46.93, according with input DNA quantity in the samples.
The highest viral loads (10^5 and 10^6 copies number of DNA / HPV 16, respectively) was detected in 40% of LGSIL cases and in 60% in HGSIL HPV 16 positive cases. (fig. 3)

High viral loads of HPV 16 were found in the group age 31 – 40, in percent of 40%. Smoking condition was not associated with high number of copies number, but 80% of oral contraceptive users had viral loads of HPV 16 of 10^6. If we compare the copies number detected in unique infections with multiple HPV type infections, we observe higher viral loads in single HPV 16 infections.

6 patients did not have any cytological test, 1 had one normal Papanicolau test, but all these women had a colposcopic suspicion of HPV infection. Theses remind the type of cervical cancer in our country, which is one opportunistic screening, whith the drawback that population at greatest risk is ofteen not screened and may mask the true risk of cancer in the population (Marks M. *et al*., 2011)

In HPV 18 positive cases, we did not found any direct correlation between viral load and the severity of cytologic result. In both situations – HPV 16 and 18 we had cases with normal Pap smear results and with high viral load of DNA/HPV. This fact is in accordance with

sensitivity of DNA/HPV tests (96%) in comparison with sensitivity of cytology (53%). (Cuzick J. *et al*., 2006)

Some authors sustain that there is one direct relationship between viral load and the CIN severity (high viral loads has the risk 60 times bigger to develop CIN III), in comparison with other papers which states that viral load is difficult to interpret because of integration of HPV genome in the host genome or because of the multiple HPV types infections. Some reports published that high viral loads of HR HPV types are associated with abnormal Pap smear / severe CIN lesion, (Peitsaro P *et al.,* 2002, van Duin M. *et al*., 2002, De Marco L *et al*., 2002, Cricca M *et al*., 2007), while others authors did not found any relations between the viral load and cervical dysplasia severity (Andersson S. *et al.,* 2005, Fontaine J *et al*. 2005).

Cricca M *et al*. established in 2007 that the viral load of HPV 16 is 1.38 x 10^6copii genom/300 ng total DNA, a value that allow detection of high grade CIN lesions.

Snijders PJ *et al*. (2009) concluded that in a cervical screening setting viral load assessment of HPV16, 18, 31 and 33 has no additive value to stratify high-risk HPV GP5+/6+-PCR-positive women for risk of >or=CIN2 or>or=CIN3.

On the other hand, two years later, Marks M. *et al*., (2011) demonstrated that repeated measurement of HPV 16 viral load may be a useful predictor in determining the outcome of early endpoints of viral infection.

Literature data are controversial because the viral load depends on the type of the pathologic product (cervical biopsy, vaginal washing) which is collected from the patients, mainly because they are containing specially cells from the surface of epithelium. Also, the risk for cervical neoplasia is associated with higher copy numbers of different HPV types and the variability in copy numbers is too great for viral load to be used as a predictor of CIN lesions. It is preferable to conclude that low viral copy numbers are associated with a low risk for developing CIN. However, further studies are warranted (WHO & IARC, 2007). Although some authors (Xi LF et *al*., 2009) found one significant association between viral load of HPV 16 and 18 with the current status smoker, in our cases we did not found one significant correlation (only 4 / 26 HPV positive cases was smoker: one had one viral load around 10^3 copies and two had the viral load around 10^5. No significant association was observed with other known HPV risk factors such as oral contraceptive use, and our findings are similar with the reports of Flores R. *et al.* 2006.

CONCLUSIONS

In a prospective study with 267 patients we found one positive correlation between dysplasia severity and viral load of HPV 16, but we have not found the same relation for HPV 18. No significant association was fount for smoking and oral contraceptive use.

Although the initial optimism regarding the clinical value of HPV viral load testing now seems misplaced, robust measurements of type-specific viral load in samples in which the integration status is also defined, could provide useful insights into the natural history of HPV infections and their relationship to disease.

The identification of more robust markers of disease progression requires a more complete understanding of the natural history of type-specific HPV infections.

REFERENCES

Andersson S, Safari H, Mints M, Lewensohn-Fuchs I, Gyllensten U, Johansson B. (2005): *Type distribution, viral load and integration status of high-risk human papillomaviruses in pre-stages of cervical cancer (CIN)*, Br J Cancer.; 92(12):2195-200.

Cricca M, Morselli-Labate AM, Venturoli S, Ambretti S, Gentilomi GA, Gallinella G, Costa S, Musiani M, Zerbini M. (2007): *Viral DNA load, physical status and E2/E6 ratio as markers to grade HPV16 positive women for high-grade cervical lesions,* Gynecol Oncol.; 106(3):549-57.

Cuzick J, Clavel C, Petry KU, Meijer CJ, Hoyer H, Ratnam S, Szarewski A, Birembaut P, Kulasingam S, Sasieni P, Iftner T. (2006) *Overview of the European and North American studies on HPV testing in primary cervical cancer screening*, Int J Cancer.;119(5):1095-101.

De Marco L, Gillio-Tos A, Bonello L, Ghisetti V, Ronco G, Merletti F. (2007): *Detection of human papillomavirus type 16 integration in pre-neoplastic cervical lesions and confirmation by DIPS-PCR and sequencing,* J Clin Virol.; 38(1):7-13.

Fontaine J, Hankins C, Mayrand MH, Lefevre J, Money D, Gagnon S, Rachlis A, Pourreaux K, Ferenczy A, Coutlée F (2005): *High levels of HPV-16 DNA are associated with high-grade cervical lesions in women at risk or infected with HIV,* AIDS; 19(8):785-94.

Flores R, Papenfuss M, Klimecki WT, Giuliano AR. (2006), *Cross-sectional analysis of oncogenic HPV viral load and cervical intraepithelial neoplasia.* Int J Cancer.; 118(5):1187-93.

Herbert A. (2009), *Cervical cancer prevention: screening*, 25th International Papillomavirus Conference, Malmo.

Marks M, Gravitt PE, Utaipat U, Gupta SB, Liaw K, Kim E, Tadesse A, Phongnarisorn C, Wootipoom V, Yuenyao P, Vipupinyo C, Rugpao S, Sriplienchan S, Celentano DD. (2011): *Kinetics of DNA load predict HPV 16 viral clearance,* J Clin Virol.; 51(1):44-9.

Meyer CJLM (2009): *HPV testing: requirements for clinical testing,* 25th International Papillomavirus Conference, Malmo.

Peitsaro P, Johansson B, Syrjänen S. (2002): *Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique,* J Clin Microbiol.;40(3):886-91.

Snijders PJ, Hogewoning CJ, Hesselink AT, Berkhof J, Voorhorst FJ, Bleeker MC, Meijer CJ. (2006): *Determination of viral load thresholds in cervical scrapings to rule out CIN 3 in HPV 16, 18, 31 and 33-positive women with normal cytology*, Int J Cancer., 119(5):1102-7.

van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ. (2002): *Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance.* Int J Cancer.; 98(4):590-5.

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Muñoz N. (1999): *Human papillomavirus is a necessary cause of invasive cervical cancer worldwide.* J Pathol.; 189(1):12-9.

WHO & IARC (2007), *IARC Monographs on the Evaluation of Carcinogenetic Risks to Humans, Human Papillomaviruses*, volume 90, Lyon, France.

Woodman CB, Collins SI, Young LS. (2007): *The natural history of cervical HPV infection: unresolved issues*, Volume 7, Nature Reviews, Cancer

Xi LF, Koutsky LA, Castle PE, Edelstein ZR, Meyers C, Ho J, Schiffman M., (2009) *Relationship between cigarette smoking and human papilloma virus types 16 and 18 DNA load,* Cancer Epidemiol Biomarkers Prev.;18(12):3490-6.

Affiliation of authors:
1 - University of Medicine and Pharmacy "Gr. T. Popa" Iaşi, Microbiology Department
2 - University of Medicine and Pharmacy "Gr. T. Popa" Iaşi, Gynecology Department
3 - University of Medicine and Pharmacy "Gr. T. Popa" Iaşi, Infectious Diseases Department
4 - Regional Centre of Public Health Iaşi.
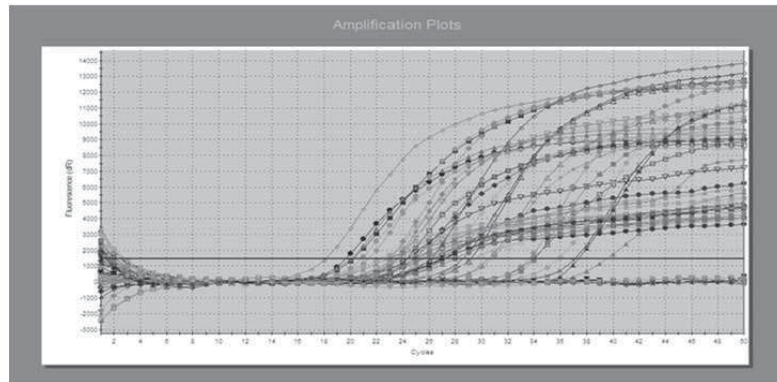
* ursuramona@yahoo.com

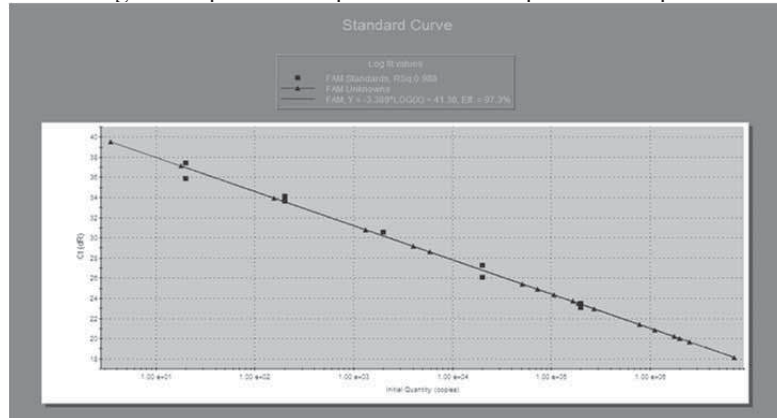Fig. 1: Amplifications plots for HPV 16 positive samples
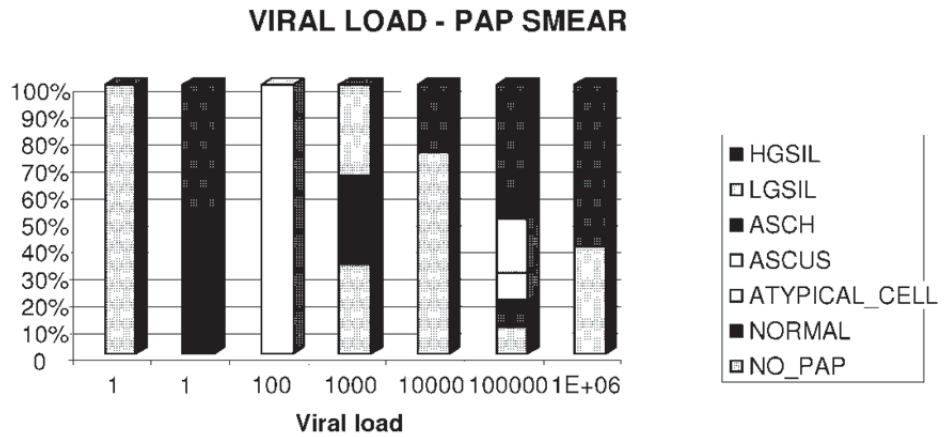

Fig. 2: Standard curve for HPV 16 positive samples

Fig. 3: Correlations viral loads of HPV 16 – Pap smear