Current Topics in Virology
Vol. 15, 2018

Short Communication

Viral clearance in a multiple infection with high-risk HPV-16 and HPV-35

Patricia Romero[1], Monserrat Balanda[2], Nicolás Vergara[2,3], Mónica Muñoz[1], Carolina González[1], Héctor San Martín[2] and Eugenio Ramírez[2,*]

[1]Unidad de Ginecología Pediátrica y Adolescente, Hospital Dr. Luis Calvo Mackenna, Santiago;
[2]Sección Virus Oncogénicos, Subdepto. de Enfermedades Virales, Instituto de Salud Pública de Chile;
[3]Dirección de Atención Primaria, Servicio de Salud Metropolitano Central, Santiago, Chile.

ABSTRACT

We report the persistence and the clearance of two high-risk human papillomaviruses (HPVs) HPV-16 and HPV-35 in a patient. The progress of HPV-16 infection was confirmed by an increased expression of viral E7 gene and cellular CDKN2A and CDKN1A genes. Simultaneously, HPV-35 clearance in this patient is shown. The expression of these viral and cellular genes could be helpful predictors of cervical lesions.

KEYWORDS: HPV, E7 mRNA, CDKN2A ($p16^{INK4a}$) mRNA, CDKN1A ($p21^{WAF1/CIP1}$) mRNA.

INTRODUCTION

HPV infections are the most common sexually transmitted disease worldwide [1]. Persistent high-risk HPV infection is the etiology of invasive cervical cancer [2]. Global epidemiology of HPV infections as well as the prevalence of genotypes in different countries is well known [3]. Furthermore, it has been shown that about a quarter of women worldwide are multiple infected with different HPV genotypes. Coinfections by multiple high-risk HPV types are associated with precancerous lesions, accelerate development of cancers, and complicate treatment outcomes [4]. However, there is only scarce knowledge about the natural history of HPV multiple infections.

*Corresponding author: eramirez@ispch.cl

The expression of early viral E6 and E7 genes has been proposed as a biomarker for evaluating the course of infection and prognosis of lesions caused by HPV [5]. Viral E7 protein promotes the degradation of cellular retinoblastoma protein (pRb), releasing the E2F factor, which induces the expression of cellular genes responsible for triggering the cell cycle progression, thus stimulating G1 to S-phase transition. Meanwhile, viral E6 protein interferes with the function of cellular p53 protein inhibiting cell apoptosis. The joint effects of E6 and E7 proteins are the key to trigger cell transformation and progress to cervical cancer. Furthermore, HPV-16 infected cells in cervical cancer have shown many deregulated cellular genes. In these cells, overexpression of CDKN2A ($p16^{INK4a}$), TOP2A, MCM2 and CDC20 mRNA, and inhibition of CDKN1A ($p21^{WAF1/CIP1}$) mRNA have been demonstrated [6, 7]. Nevertheless, increased expression of $p21^{WAF1/CIP1}$ protein has been shown in advanced disease stage [8, 9]. CDKN2A gene is a putative oncosupressor gene encoding two unrelated proteins: $p16^{INK4a}$ and p14ARF. $p16^{INK4a}$ protein regulates pRb-dependent G1 arrest by inhibiting the phosphorylation of pRb by the cyclin-dependent kinases CDK4 and CDK6 [10, 11]. p14ARF protein blocks MDM2-induced p53 degradation resulting in an increase in p53 levels and cell cycle arrest in both G1 and G2/M [10, 11]. The $p21^{WAF1/CIP1}$ protein is a wild-type p53 inducible protein that inactivates the cyclin/cdk complexes

blocking the cell cycle progression in the G1–S transition with the consequent inhibition of apoptosis [12]. However, $p21^{WAF1/CIP1}$ can independently promote oncogenesis of its anti-apoptotic activity by promoting the assembly of complexes of cyclin D with CDK4 or CDK6 without inhibiting their kinase activity [13]. The level of expression of these cellular genes has been suggested as biomarkers for the prognosis of progress of viral infection. Here we describe a clinical case in which the progress of genital lesions caused by multiple infection with two high-risk HPV genotypes was molecularly evaluated. To study the progress of infection, we evaluated the viral load and levels of mRNA of viral E7 gene, and cellular CDKN2A ($p16^{INK4a}$) and CDKN1A ($p21^{WAF1/CIP1}$) genes.

CASE REPORT

In this study we present the case of an asymptomatic woman of 25 years of age whose boyfriend had genital condyloma. She underwent bone marrow transplantation for severe aplastic anemia when she was 12 years old. She was not receiving inmunosupresive therapy at the beginning of the gynecological follow up. She has been clinically evaluated since October 2014 at the gynecology department of the East Reference Hospital of Metropolitan Health Service (Dr. Luis Calvo Mackenna Hospital) Santiago. The external genital exam was normal, wherein a cervical ectropion was visualized in the cervix. Pap test done on June 2014 showed a normal cytology. Also, this patient had normal parameters according to hemogram analysis. Three swabs of uterine cervix were obtained during the follow-up (October 2014, May 2015 and July 2015) for virological diagnosis and molecular analysis. Serological assays for anti-HIV and anti-*T. pallidum* were negative. She was referred to an oncology gynecologist for follow-up and study in 2015. Colposcopy showed a cervical lesion, which was biopsied, and the report confirmed a L-SIL (Low grade squamous intraepithelial lesion) in January 2016. Later, she was studied by means of colposcopic exams every six months. This patient agreed to participate in the study and signed an informed consent form. This case study was done according to the guidelines of the National Cancer Program of Chile.

Molecular biology methods

Molecular viral diagnosis and HPV typing were performed by real time PCR (Polymerase Chain Reaction) and PCR-RLB (Reverse Line Blotting) of L1 HPV gene, respectively [14]. PCR-RLB was performed with equal amount of purified DNA from each sample to normalize the hybridization signal. Quantitation of viral E7 gene by real time PCR was made to determine the viral load of HPV-16. A standard curve with cloned E7 plasmid was made for quantitative real PCR, using SiHa cells (infected with HPV-16) as a control positive and β-globin gene as reference. Viral load was measured as the ratio of copy number of HPV-16 E7 gene/copy number of β-globin gene. Expression of E7, CDKN2A and CDKN1A mRNA was determined by real time RT-PCR with a standard curve for each gene with SiHa cells. Relative quantitation was done using the comparative threshold cycle (ΔΔCt) formula with HPRT as the endogenous housekeeping gene. Standard curves for the values of E7, CDKN2A and CDKN1A mRNA and HPRT mRNA were generated using cDNA from SiHa cells. All assays were carried out in triplicate and the average value was used for calculations.

RESULTS

In the first sample (October, 2014) multiple infection with HPV-16 and HPV-35 was determined (Figure 1). This multiple infection was also detected seven months later, when the second sample was taken (May, 2015). Similar HPV-16 reactivity was detected in both samples (Figure 1). However, the level of HPV-35 reactivity in the second sample was found to be strongly decreased compared to the first sample. In the third sample, HPV-16 reactivity was similar to the other two samples whereas HPV-35 showed almost negative reactivity (Figure 1). These results suggest a persistent HPV-16 infection and a simultaneous clearance of HPV-35 infection.

The viral loads of HPV-16 were 3.25, 2.96 and 3.61 in the first, second and third sample, respectively. These results suggested similar values of HPV-16 viral load during the follow-up (Table 1). Furthermore, these findings were consistent with those detected by PCR-RLB,

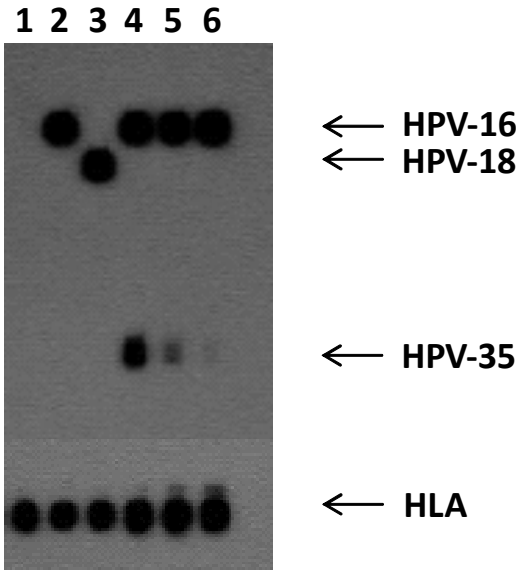

Figure 1. Detection of L1 HPV gene among three cervical samples from a multiple infected woman by PCR-RLB assay. Representative PCR-RLB assay of DNA (50 ng) from cervical samples. Lanes are: 1. Negative control (K-652 cells), 2. HPV-16 positive control (SiHa cells), 3. HPV-18 positive control (HeLa cells), 4. First sample of patient (October, 2014), 5. Second sample of patient (May, 2015), 6. Third sample of patient (July, 2015). HLA: the hybridization signal of this internal control gene for each sample and the control. Arrows show the hybridization signals of L1 HPV-16, L1 HPV-18, L1 HPV-35 and HLA genes.

Table 1. Changes in E7, CDKN2A ($p16^{INK4a}$), and CDKN1A ($p21^{WAF1/CIP1}$) mRNA and HPV viral load in a multiple infected patient. Viral load was determined as HPV E7 gene copies/β-globin gene copies. Expression of E7, CDKN2A and CDKN1A mRNA was determined by real time RT-PCR and relative quantitation (ΔΔCt) methods using HPRT as the endogenous housekeeping gene. Data are reported as means ± SD levels. 1. First sample (October, 2014), 2. Second sample (May, 2015), 3. Third sample (July, 2015).

Sample n°	ΔΔCt E7 mRNA	ΔΔCt $p16^{INK4a}$ mRNA	ΔΔCt $p21^{WAF1/CIP1}$ mRNA	HPV E7 gene Viral load
1	1.02 ± 0.25	0.90 ± 0.21	0.55 ± 0.30	3.25 ± 0.27
2	1.07 ± 0.13	0.98 ± 0.14	0.95 ± 0.18	2.96 ± 0.35
3	1.93 ± 0.17	2.19 ± 0.16	0.83 ± 0.19	3.61 ± 0.23

where the three samples showed similar reactivity with HPV-16 probe (Figure 1).

Furthermore, the expression of E7 viral gene was similar in the first and second sample. However, the level of this mRNA showed a significant increase of 1.93 times in the third sample (Table 1). Similarly, CDKN2A (16^{INK4a}) mRNA levels were 0.90 and 0.98 in the first and second sample, respectively. But, it showed a significant increase of 2.19 times in the third sample (Table 1). Finally, the level of CDKN1A ($p21^{WAF1/CIP1}$) mRNA was similar in all the samples. Its gene expression fluctuated between 0.55 and 0.95 (Table 1). This variation was not significant.

DISCUSSION

The reactivity of L1 gene obtained by PCR-RLB simultaneously showed a clearance of the HPV-35 DNA and the persistence of the HPV-16 DNA during the follow-up. The viral load of HPV-16

showed similar levels in the three samples according to the reactivity detected with HPV-16 L1 gene by PCR-RLB. Consequently, these findings suggest a clearance of HPV-35 and a persistence of HPV-16 in this patient during the studied period of time (eight months). Moreover, the level of HPV-16 E7 mRNA increased significantly during the follow-up. This increased level of E7 transcript should suggest an increased synthesis of E7 viral protein. Nevertheless, our study represents a limited approach because we did not quantify E7 protein. High levels of E7 protein and mRNA have been suggested as biomarkers of the progress of HPV infection [15, 16]. We could detect a variation in the level of HPV-16 E7 mRNA from 1.02/1.07 to 1.93 units of relative quantity during the 8 months of follow-up in this patient. The increased expression of E7 mRNA was coincident with the clinical evolution from an asymptomatic stage to a low grade squamous intraepithelial lesion. This finding would support E7 mRNA as a predictor of the progress of HPV infection.

Both cellular transcripts, 16^{INK4a} and $p21^{WAF1/CIP1}$, have been suggested as biomarkers of high-grade cervical lesions [17, 18]. An increased expression of CDKN2A gene was detected in this patient during the follow-up. However, CDKN1A mRNA levels were similar during the follow-up period. These findings suggest that increased levels of CDKN2A cellular transcripts could be a predictor for the development of lesions. To date, the only widely recommended test for triage of HPV-infected women and reducing the number of colposcopies in HPV-based screening is cytology. However, the sensitivity of cytology is affected by multiple factors such as quality of sampling, staining technique, training of professional staff, etc. Consequently, we suggest that both viral E7 and cellular CDKN2A (16^{INK4a}) mRNAs could be helpful predictors for the progress of HPV infection and development of lesions in high-risk HPV-infected women.

CONCLUSION

The persistence of the HPV-16 infection and the clearance of the HPV-35 were demonstrated in a patient after 8 months of follow-up. Increase in E7 and CDKN2A mRNA levels was determined in cervical samples before the development of cervical lesion. Consequently, both mRNA levels could serve as useful biomarkers of the progress of HPV infection.

CONFLICT OF INTEREST STATEMENT

None to declare.

REFERENCES

1. Muñoz, N., Bosch, F. X., de Sanjosé, S., Herrero, R., Castellsagué, X., Shah, K. V., Snijders, P. J. and Meijer, C. J. 2003, N. Engl. J. Med., 348, 518.
2. Moscicki, A. B., Schiffman, M., Burchell, A., Albero, G., Giuliano, A. R., Goodman, M. T., Kjaer, S. K. and Palefsky, J. 2012, Vaccine, 30, F24.
3. Bruni, L., Barrionuevo-Rosas, L., Albero, G., Serrano, B., Mena, M., Gómez, D., Muñoz, J., Bosch, F. X. and de Sanjosé, S. 2017, ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World. Summary Report 27 July 2017. [Date Accessed] http://www.hpvcentre.net/summaryreport.php
4. Trottier, H., Mahmud, S., Costa, M. C., Sobrinho, J. P., Duarte-Franco, E., Rohan, T. E., Ferenczy, A., Villa, L. L. and Franco, E. L. 2006, Cancer Epidemiol. Biomark Prev., 15, 1274.
5. Bodily, J. and Laimins, L. 2011, Trends in Microbiol., 19, 33.
6. Santin, A. D., Zhan, F., Bignotti, E., Siegel, E. R., Cané, S., Bellone, S., Palmieri, M., Anfossi, S., Thomas, M., Burnett, A., Kay, H. H., Roman, J. J., O'Brien, T. J., Tian, E., Cannon, M. J., Shaughnessy, J. Jr. and Pecorelli, S. 2005, Virology, 331, 269.
7. Wong, Y., Cheung, T., Tsao, G., Lo, K. W., Yim, S. F., Wang, V. W., Heung, M. M., Chan, S. C., Chan, L. K., Ho, T. W., Wong, K. W., Li, C., Guo, Y., Chung, T. K. and Smith, D. I. 2006, Int. J. Cancer, 118, 2461.
8. Cheung, T. H., Lo, K. W., Yu, M. M., Yim, S. F., Poon, C. S., Chung, T. K. and Wong, Y. F. 2001, Cancer Lett., 172, 93.

9. Bae, D. S., Cho, S. B., Kim, Y. J., Whang, J. D., Song, S. Y., Park, C. S., Kim, D. S. and Lee, J. H. 2001, Gynecol. Oncol., 81, 341.
10. Quelle, D. E., Zindy, F., Ashmun, R. A. and Sherr, C. J. 1995, Cell, 83, 993.
11. Sano, T., Masuda, N., Oyama, T. and Nakajima, T. 2002, Pathol. Int., 52, 375.
12. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W. and Vogelstein, B. 1994, Cancer Res., 54, 1169.
13. Gartel, A. L. 2006, Mol. Cancer Ther., 5, 1385.
14. Balanda, M., Quiero, A., Vergara, N., Espinoza, G., Martín, H. S., Rojas, G. and Ramírez, E. 2016, Med. Microbiol. Immunol., 205, 585.
15. Hesselink, A. T., Berkhof, J., van der Salm, M. L., van Splunter, A. P., Geelen, T. H., van Kemenade, F. J., Bleeker, M. G. and Heideman, D. A. 2014, J. Clin. Microbiol., 52, 890.
16. Depuydt, C. E., Jonckheere, J., Berth, M., Salembier, G. M., Vereecken, A. J. and Bogers, J. J. 2015, Cancer Med., 4, 1294.
17. Manawapat-Klopfer, A., Thomsen, L. T., Martus, P., Munk, C., Russ, R., Gmuender, H., Frederiksen, K., Haedicke-Jarboui, J., Stubenrauch, F., Kjaer, S. K. and Iftner, T. 2016, Am. J. Cancer Res., 6, 1524.
18. Gustinucci, D., Giorgi Rossi, P., Cesarini, E., Broccolini, M., Bulletti, S., Carlani, A., D'angelo, V., D'amico, M. R., Di Dato, E., Galeazzi, P., Malaspina, M., Martinelli, N., Spita, N., Tintori, B., Giaimo, M. D. and Passamonti, B. 2016, Am. J. Clin. Pathol., 145, 35.