Original Article

Absolute Quantification of Gene E7 in Cervical Samples of Women Infected by HPV Using Real Time PCR

Jussara de Sousa Ribeiro Bettini,[1] Renata Toscano Simões,[1] Alessandra Cristina Gomes Ruiz,[2] Benedito Antonio Lopes da Fonseca,[2] Aguinaldo Luis Simões,[3] Maria do Carmo Tremitão Canas,[3] Geraldo Duarte,[4] Silvana Maria Quintana,[4] Ciro Dresch Martinhago,[5] Armindo Antonio Alves,[6] Ana Maria Rocha,[1] Edson Garcia Soares[1]

1 Department of Pathology, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

2 Department of Clinical Medicine, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

3 Department of Genetics, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

4 Department of Gynecologi and Obstetrics of Clinical Hospital, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

5 Medical Diagnosis RDO, São Paulo, Brazil

6 Herminio Ometto Foundation, Araras, São Paulo, Brazil

Abstract

Objective: High grade oncogenic types of human papillomavirus (HPV), especially HPV16 and HPV18, possess a gene called E7, which acts on genes that regulate cell growth, promoting development of pre-neoplastic lesions that can lead to invasive carcinomas. The absolute quantification of this gene in cervical samples of HPV-infected women may contribute for better understanding the evolution of these lesions induced by the virus. **Methods:** We collected 60 cervicovaginal smears of women infected by HPV with or without uterine cervical squamous intraepithelial lesion, SIL) and 10 samples of women with no HPV infection or SIL. The absolute quantification of gene E7 was performed by Realtime PCR using specific primers and probes. **Results:** Samples infected by HPV16 have a higher number of gene E7 copies when compared to samples infected by HPV18. In the HPV18 group it was observed that those obtained from patients with low or high grade squamous intraepithelial lesions (HSIL) or invasive cervical cancer presented significantly higher concentrations of gene E7 when compared to patients with no cervical lesions. The number of gene E7 copies was higher in the group infected by HPV16 than by HPV18. In spite of that, there was no difference in the number of gene E7 copies in samples infected by HPV16 with or without SIL. **Conclusions:** Among the samples with HPV18, the number of copies of gene E7 was higher in the group with cervical lesions, and no differences were found for SIL, HSIL or invasive cancer patients.

Keywords: Papillomavirus e7 proteins. Infection. Cervix. HPV.

Introduction

Epidemiologic and molecular studies done in the last 20 years confirm that infection by certain types of human papillomavirus (HPV) is a precursor event of cervical neoplasia, for the identification of the HPV genoma is positive in the majority of these tumors.[1-3] Nevertheless, little is known about the risk determinants of HPV persistent infection, since patients with persistent infection by oncogenic types of HPV present higher susceptibility to develop cervical neoplasia when compared to those infected temporarily.[4-7] More than 150 types of HPV have been described and one knows that

Correspondence
Jussara de Sousa Ribeiro Bettini
Rua Paul Harris, 200 (114)
13600230, Araras, Brazil
Telephone: 55 19 35431400 (269)
E-mail: jussarabettini@uniararas.br

HPV16 and 18 types are the ones most associated to SIL (squamous intraepitelial lesions) and invasive cancer.[8]

It is also known that all cell alterations induced by HPV infection are caused by the combination of viral genes function. At this point, one knows that gene E7 contributes directly to abnormal cell growth typical of SIL and carcinomas. Besides, the number of viral DNA present in the tumor may represent an important aspect of the disease.[9-10] Researchers ask whether gene quantification in cervical samples of women infected by HPV is a predictor factor of the evolution of lesions induced by this virus.

Real Time PCR is an efficient and objective technique of molecular biology that detects and quantifies DNA target sequence in a period of two or three hours, whereas classic PCR needs at least four hours to detect the same sequence without quantifying it. This new PCR technique uses only fluorogenic probes, increasing the specifity of the reaction. The TaqMan probe, marking at the end 5' with a fluorochrome reporter (FAM) and at the end 3' with a fluorochrome quencher (TAMRA), anneals the marked sequence between forward and reverse primers, generating a substrate that is clived by 5' of Taq DNA polimerase. This process just occurs if the target sequence is amplified.[11-12] Because of these data, this study had the purpose to evaluate the concentration of gene E7 among women infected by HPV types 16 and 18 with LSIL (low squamous intraepitelial lesions) or HSIL (high squamous intraepitelial lesions) and without SIL.

Casuistic and Methods

Patients and sample collection

We analyzed 106 cervicovaginal samples of women obtained from patients treated in the Division of Infectious Diseases of Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, University of São Paulo. All patients were informed about the research and signed the consent form. After HPV diagnosis and HPV16 and HPV18 typing, patients were divided in three groups: group A (30 women with HPV without SIL), group B (30 women with HPV with SIL or invasive cancer) and group C, negative control (10 women without infection by HPV and without SIL). Samples infected by two types HPV16 and 18 were excluded.

Polymerase Chain Reaction

DNA extraction from the samples was performed as described by Higuchi[13] PCR reaction for the diagnosis and HPV typing was performed according to the technique described by Walboomers et al.[6] and Bettini et al.[14]

Real Time PCR

Samples were submitted to Real Time PCR for absolute quantification of gene E7 in patients infected by HPV 16 and 18. DNA was quantified by Gene Amp 5700 (Applied Biosystems, Foster City, Calif.) system of DNA sequence detection. PCR mix consisted of 3ul of DNA, 12.5ul of 2X universal master mix (Applied Biosystems, Foster City, Calif.), 8.75ul of sterile water, 0.5ul of primer forward (10uM), 0.5ul of primer reverse (10uM) and 0.5 ul of TaqMan TAMRA probe (5uM) with a total volume of 25ul. Primer sequences were selected using Primer Express software (version 1.0) from Applied Biosystems) (Table 1).

Amplification was performed at 50°C for 2 minutes and initial denaturation for 10 minutes at 95°C, followed by 55 cycles of 15 seconds at 94°C and a pause of 1 minute at 60°C. The standard curve was obtained by amplification in several dilutions of two clones of HPV16 and HPV18 pBR322 vector (donated by Dr. Ethel Michelle de Villiers, German Cancer Center, Heidelberg, Germany).

Standard Curves

Standard curves corresponding to HPV types 16 and 18 were built from several dilutions, performed in two clones containing vector PBR322 and the complete genoma of HPV16 and HPV18, using ultrapure DNA of *Escherichia coli*. With titration, we obtained 20,000, 6,000, 2,000, 600, 200, 60, 20, 6 and two copies / microliter of samples for each type of HPV.

Real Time PCR results

Analysis was performed to see the relationship between the number of DNA and Ct (threshold cycle-beginning of the fluorescence detection) because it is known that the higher the Ct, the higher the DNA number is.

Table 1 – Specific primers and probes

HPV16E7-96F	AGAGGAGGAGGATGAAATAGATGGT
HPV16E7–182R	CACTTGCAACAAAACGTTACAATATTG
HPV16E7-122T	6FAMCAGCTGGACAAGCAGAACCGGACATAMRA
HPV18E7-2F	TGCATGGACCTAAGGCAACA
HPV18E7-78R	TAGAAGGTCAACCGGAATTTCATT
HPV18E7-23T	6FAMTGCAAGACATTGTATTGCATTTAGAGCCCCTAMRA

Statistical Analysis

Statistical analysis was done using Wilcoxon rank-sum test, based on the amount of copies of Gen E7 in the samples, to verify the difference among the group of positive HPV patients with SIL, the positive group without SIL, the group of samples infected just by HPV16 and the group of samples infected just by HPV18 and the negative patients group without SIL, with a significance of $p < 0.05$.

Results

Both samples of HPV16 and HPV18 had Ct from 12 to 31. The coefficient correlation of HPV16 and 18 were respectively 0.988826 and 0.985172, indicating the good quality of the standard samples. The minimal limit detection was 20 copies/microliter indicating the good sensitivity and quality of the assay. No copies of gene E7 were observed in the samples of the negative HPV group without lesions. In spite of that, there were differences between samples infected by HPV16 and HPV18 ($p<0.0001$). In HPV16 samples, the number of copies of the gene E7 was higher than that of samples infected by HPV18, an average of 499.188.235, 652.030, respectively (Table 2).

Regarding samples with HPV18 there were significant differences between groups with and without SIL ($p=0.0129$). There was a higher number of gene E7 copies in samples HPV18 with SIL (Figure 1). Nevertheless, between the groups of women positive to HPV16 with and without SIL there were no significant differences ($p = 0.4173$).

Discussion

In agreement with Boer et al.[15] and most authors,[16-18] HPV is the main cause of uterine cervix cancer and the presence of types 16 and 18 in cervical mucosa increases the progression of LSIL to a neoplastic lesion. One can not forget that a high percentage of women with HPV develop only transitory warts or get rid of the virus without any lesion.[19]

Mcmurray et al.[9] state that all alterations induced by HPV are caused by a combination of viral genes E1, E2, E4, E5, E6 and E7. Among other functions gene E7 affects genes transcription involved in the progression of cell cycle and differentiation inducing its proliferation, immortalization and transformation.

HPV is able to stimulate G1 progression and the start of phase S, besides replicating its own genoma inside the cell. Probably when they reach their goal they affect

Table 2 – Average number of copies of E7 gene, quantified in the samples infected by HPV: with SIL, without SIL, samples infected just by HPV16 and samples infected just by HPV18

Samples with SIL	Samples without SIL	Samples HPV16	Samples HPV18
773.238.709	743.886.893	499.188.234,80	652.029,93

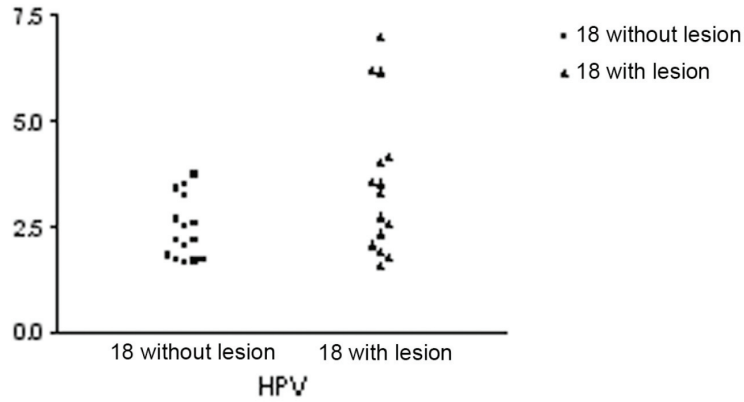

Figure 1 - Relationship between samples of HPV18 subtype with and without SIL

cell cycle in many ways. The disarrangement caused by the oncogenic virus may produce chromosomal alterations that predispose to malign transformations. Because these alterations do not occur with non oncogenic types, the infection by these viral types allows for satisfactory results to virus with minimal effects to epithelial cells. The difference between oncogenic and non oncogenic virus is for example the interaction between protein E7, produced by the action of gene E7 and protein Rb. The affinity of protein Rb with protein is higher to E7 of oncogene E7 than to the same non-oncogenic genes.[9,19]

Real Time PCR uses two general methods to quantify the amplicom: Taqman and Syber green. In 2002, Nagao et al.[20] developed a protocol to quantify DNA of HPV 16 through Real Time PCR, using Syber Green molecular probes and obtained good results, due to the fact that the protocol was well standardized. Morberg et al.[4] stated that TaqMan is more specific and sensitive than Syber Green. TaqMan probes use the activity of exonuclease fluorogenic 5' of Taq polimerase to measure the number of marked sequences in DNA samples, annealing to the internal region of PCR product. When polymerase amplifies the sequence to which Taqman probe is connected, the activity of exonuclease fluorogenic 5' clives the probe. This finalizes the activity of the quencher and the reporter starts to emit fluorescence, which increases each cycle proportionally, due to probe clivage. The accumulation of amplified products is detected through the increase of reporter fluorescence. TaqMan probes originate specific amplifications because the clivage occurs only if the probe hybridizes the target. Thus, this method is sensitive and more specific than Syber Green. Real time PCR with molecular beacons possesses reporters that adapt to the shape of tweezers, stay free in the solution to produce fluorescence and the quencher approximates to produce the fluorescence to be detected. However, the proximity of reporter and quencher inhibits reporter fluorescence.[12] In this analysis, it was used Taqman primers and probes for HPV16 and HPV18gene E7 quantification. The results were satisfactory, mainly as regards the technique precision, although this is a high-cost procedure.

In several reports performed with Real Time PCR, there were obtained dilutions of 10^{-1} to 10^{6} copies of DNA using sterile water.[21-22] In this investigation, there were obtained HPV 16 and 18 dilutions plasmids in DNA ultra pure of *Escherichia coli*, to obtain 20.000, 6.000, 2.000, 600, 200, 60, 20, 6 and 2 copies of HPV/microliter. It was observed an amplification until 20 copies, without amplification in the samples with 6 and 2 copies/microliter, which proves the high sensitivity of this method.

Initially it was believed that there would be a difference among the quantities of gene of women carrying HPV without SIL and infected women with HPV with SIL or invasive cancer. Nevertheless, in this study there were observed differences only in subtype HPV18, and samples of women with SIL or invasive cancer presented a higher number of copies than women without SIL.

Oncogenic transcription may be considered a risk for the development of SIL and its progression to cervical cancer.[23] E7 oncogenes possess an important transcriptional activity in the development and maintenance of cervical carcinogenesis found in squamous cells. HPV 16 has two receptors that generate three different transcriptions, which favors the production of more proteins than HPV18, which possess only one receptor generating only

two transcriptions.[24] Biedermann et al.[10] state that the number of viral DNA present in the tumor may represent an important aspect to the course and aggressiveness of the disease. Our data confirm the author statement, showing that viral type can also influence the course of the disease. In our samples, those infected by HPV16 have a greater number of gene E7 copies when compared to those infected by type 18, which lead us to understand why this type of HPV is more associated to the evolution of SIL to uterus cancer, and also to be considered by authors the more virulent type.[15,23,25] We also conclude that in our samples there is no significant difference between patients positive to HPV16 with or without SIL induced by the virus, regarding the number of gene E7, but there is a significant difference among samples with HPV18, mainly those with SIL which presented a higher number of copies of this gene.

Acknowledgements

We are grateful to Dr. Ethel Michelle de Villiers, German Cancer Center, Heidelberg, Germany for the clones; Andrea Trevisan, Ludwig Institute for Cancer Research, São Paulo Brasil, for technical assistance, Adrianne Christine Palanch and Gislaine Maria Fontanetti Bortolotti, Herminio Ometto Foundation Araras – São Paulo – Brazil, for technical assistance and Capes and Fapesp for financial support.

References

1. Túlio S, Pereira LA, Neves FB, Pinto AP. Relação entre a carga viral de HPV oncogênico determinada pelo método de captura híbrida e o diagnóstico citológico de lesões de alto grau. J Bras Patol Med Lab 2007;43:31-5.
2. Lima CA, Palmeira JAV, Cipotti R. Fatores associados ao câncer do colo uterino em Propriá, Sergipe, Brasil. Cad Saúde Pública 2006;22:2151-6.
3. Nonnenmacher B, Breitenbach V, Villa LL, Prolla JC, Bozzetti MC. Identificação do papilomavírus humano por biologia molecular em mulheres assintomáticas. Rev Saúde Pública 2002;36: 95-100.
4. Morberg 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;41:3221-28.
5. Bezerra SJS, Gonçalves PC, Franco ES, Pinheiro AKB. Women's profile with cervical lesions for HPV as for the risk factors for cancer of cervix DST. J Bras Sex Transm Dis 2005;17:143-8.
6. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cancer worldwide. J Pathol 1999;189:12-9.
7. Voglino G, Poso F, Privitera S, Parisio F, Ghiringhello B, Gordini G, Chiara G, Massobrio M, Fessia L. The role of human papillomavirus in cyto-histological practice: distribution and prevalence of high-risk strains (16, 18, 31, 33, and 35) in intraepithelial lesions and neoplasia of the uterine cervix. Pathologica 2000;92:516-23.
8. Martinez I, Wang J, Hobson KF, Ferris RL, Khan SA. Indentification of differentially expressed genes in HPV – positive and HPV – negative oropharyngeal squamous cell carcinomas. Eur J Cancer 2007;43:415-32.
9. McMurray HR, Nguyen D, Westbrook TF, McAnce DJ. Biology of human papillomaviruses. Int J Exp Pathol 2001;82:15-33.
10. Biedermann K, Dandachi N, Trattner M, Vogl G, Doppelmayr H, Moré E, Staudach A, Dietze O, Hauser-Kronberger C. Comparison of real-time PCR signal amplified in situ hybridization and comventional PCR for detection and quantification of human papillomavirus in archival cervical cancer tissue. J Clin Microbiol 2004;42:3758-65.
11. Grove DS. Quantitative real time polymerase chain reaction for the core facility using TaqMan and the perkin-elmer/applied biosystems division 7700 sequence detector. J Biomol Tech 1999;10:11-6.
12. Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. Nucleic Acids Res 2002;30:1292-305.
13. Higuchi R. Simple and rapid preparation of samples for PCR. In: Erlich HA, editor. PCR technology. New York: Stockton Press; 1989. p.31-8.
14. Bettini JSR, Soares EG, Duarte G, Simões RT, Simões AL. PCR diagnosis of HPV in cervical biopsies of CIN and invasive neoplasia formerly diagnosed as HPV negative. Acta Cytol 2003;47:545-9.
15. De Boer MA, Peters LA, Aziz MF, Siregar B, Cornain S, Vrede MA, Jordanova ES, Fleuren GJ. Human papillomavirus type 18 variants: histopathology and E6/E7 polymorphisms in three countries. Int J Cancer 2003;114:422-5.
16. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet 2007;370:890-907.
17. Menzo S, Marinelli K, Bagnarelli P, Rolla S, Clementi M. Human papillomavirus infections: new perspectives for prevention and treatment. New Microbiol 2007;30:189-212.
18. Sussman AL, Helitzer D, Sanders M, Urquieta B, Salvado M, Ndiaye K. HPV and cervical cancer prevention counseling with younger adolescents: implications for primary care. Ann Fam Med 2007;5:298-304.
19. O'Brien PM, Saveria Campo M. Evasion of host immunity directed by papillomavirus-encoded proteins. Virus Res 2002 88:103-17.
20. Nagao S, Yoshinouchi M, Miyagi Y, Hongo A, Kodama J, Itoh S, Kudo T. Rapid and sensitive detection of physical status of human papillomavirus type 16 DNA by quantitative real-time PCR. J Clin Microbiol 2002;40:863-67.
21. Dorak MT. Real time PCR. J Mol Biol 2002;45:33-40.
22. Glahder JA, Hansen CN, Vinther J, Madsen BS, Norrild B. A promoter within the E6 ORF of human papillomavirus type 16 contributes to the expression of the E7 oncoprotein from a monocistronic mRNA. J Gen Virol 2003;84:3429-41.
23. Sotlar K, Stubner A, Diemer D, Menton S, Menton M, Dietz K, Wallwiener D, Kandolf R, Bültmann B. Detection of high-risk human papillomavirus E6 and E7 oncogene transcripts in cervical scrapes by nested RT-polymerase chain reaction. J Med Virol 2004;74:107-16.
24. Scheurer ME, Dillon LM, Chen Z, Follen M, Adler-Storthz K. Absolute quantitative real-time polymerase chain reaction for the measurement of human papillomavirus E7 mRNA in cervical cytobrush specimens. Infect Agent Cancer 2007;2:8.
25. Sathish N, Abraham P, Peedicayil A, Sridharan G, Shaji RV, Candí G. E2 sequence variations of HPV 16 among patients with cervical neoplasia seen in the Indian subcontinent. Gynecol Oncol 2004;95:363-9.