TYPES AND VARIANTS OF HUMAN PAPILLOMAVIRUS IN PATIENTS WITH CERVICAL CANCER SUBMITTED TO RADIOTHERAPY[1]

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ABSTRACT

INTRODUCTION: high risk HPV infections and variants presence has been associated to increase the risk of cervical cancer. However, there are few studies that analyze the presence of them in patients with cervical cancer before and alter radiotherapy treatment. OBJECTIVES: to analyse the human papilloma virus presence and E7/HPV16 variants in 60 women with cervical cancer before and after radiotherapy. MATERIALS AND METHODS: HPV detection and typing were based on a GP5+/GP6+ PCR – Enzyme immune assay. E7/HPV16 variants were detected by PCR -Single strand conformation polymorphism (SSCP) and confirmed by direct sequence. RESULTS: before radiotherapy, 50/60 patients (83.3%) were HPV positive and HPV16 (53.3%) was the most prevalent type. After 3 months of radiotherapy, 55 patients attended to consult; of them, 19 (34.6%) were HPV positive, this decrease in the HPV detection was significant ($p<0.0005$). The E7/HPV16 analysis showed that 20 samples (62.5%) amplified before radiotherapy, 18 of them (90%) had identical SSCP pattern to the prototype and 2 showed a different SSCP pattern. The sequence of these two samples showed silent mutations at nt. 732 (T-to-C), 789 (T-to-C) and 795 (T-to-G). After radiotherapy, the was not detection of new mutations, 6 patients showed persistent HPV16

TIPOS Y VARIANTES DEL VIRUS DEL PAPILOMA HUMANO EN PACIENTES CON CÁNCER CERVICAL SOMETIDOS A RADIOTERAPÍA

RESUMEN

INTRODUCCIÓN: los virus del papiloma humano de alto riesgo y la presencia de algunas variantes han sido asociados con el incremento del riesgo de cáncer cervical. Sin embargo, existen muy pocos estudios en los que se haya analizado su presencia en pacientes antes y después de ser remitidos a radioterapia. OBJETIVOS: analizar la presencia del virus del papiloma humano y de variantes de E7/VPH16 en 60 mujeres con cáncer cervical antes y después del tratamiento con radioterapia. MATERIALES Y MÉTODOS: la detección y tipificación del VPH antes y después de radioterapia se basó en un GP5+/GP6+ PCR –Inmunoensayo enzimático. Variantes de E7/VPH16 se detectaron mediante PCR -Polimorfismo conformacional de cadena sencilla y confirmadas por secuencia directa. RESULTADOS: antes de radioterapia, 50 de 60 pacientes (83.3%) fueron positivas para VPH, siendo el VPH16 (53,3%) el más prevalente. Después de radioterapia, de 55 pacientes que asistieron, 19 (34,6%) fueron VPH positivas.

[1] The project received financial support from the Instituto Nacional de Cancerología in Bogotá, Colombia. Project "Investigación para el uso de sondas frías para el estudio-relación virus del papiloma humano y cáncer de cervix". Cod 4103038-1.
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[3] The authors do not have commercial or other associations that might pose a conflict of interest.

Recibido: Enero 15 de 2007 - Aceptado: Febrero 1 de 2007
Biosalud, Volumen 6, Enero - Diciembre, 2007. págs. 45 - 57

infection with the same SSCP pattern to the prototype, and samples that initially showed a different SSCP pattern were negative to E7/HPV16 after radiotherapy. CONCLUSION: few E7/HPV16 variants were detected before radiotherapy and it seems that the treatment did not cause mutations in this gene.

Key words: DNA Probes HPV, cervical cancer, mutation, radiotherapy, Polymerase Chain Reaction, DNA sequence, Colombia.

Esta disminución en la detección del VPH fue estadísticamente significativa (p<0,0005). El análisis de E7/VPH16 mostró que 20 muestras (62,5%) amplificaron para este gen antes de radioterapia, 18 (90%) de estas muestras tuvieron un patrón de corrido por SSCP idéntico al patrón prototipo y dos muestras mostraron un patrón de corrido diferente. La secuencia de estas dos muestras mostró mutaciones silenciosas en las posiciones nt 732 (T-to-C), 789 (T-to-C) y 795 (T-to-G). Después de radioterapia, a ninguna muestra se le detectó mutaciones, seis pacientes mostraron infección persistente por VPH16 y tuvieron por SSCP un patrón de corrido similar al prototipo. Las muestras que inicialmente mostraron un patrón de corrido diferente fueron negativas para E7/VPH16 después de radioterapia. CONCLUSIONES: se detectaron pocas variantes en E7/VPH16 antes de la radioterapia y parece que este tratamiento no causa mutaciones en el gen.

Palabras clave: Sondas ADN HPV, cáncer cervical, mutación, radioterapia, Reacción en Cadena de la Polimerasa, secuencia de bases, Colombia.

INTRODUCTION

Human papilloma virus (HPV) is the main risk factor involved in the pathogenesis of genital tumours, including cervical squamous cell cancers, adenocarcinomas, vulvar carcinomas and penile cancers (45).

HPV genotypes have been grouped as high risk and low risk types on basis of their prevalence rates in pre-malignant cervical lesions and cervical invasive cancer respectively.

HPV16 is the most commonly detected type in genital infections, persists longer and it is the main virus detected in invasive cervical cancer (3,26,32,33,46). Furthermore, detection of certain HPV16 variants has been associated with a higher risk of high grade squamous intraepithelial lesions and invasive cervical cancer in several studies (12,28,40,43,49).

E6 and E7 oncogenic proteins, interact and inhibit the activities of critical components of cell cycle regulatory systems, in particular E6 with p53 and E7 with Rb (24,30,31,36). Moreover, sequence variations in HPV proteins may affect virus assembly (25), immunologic recognition by the host (6,10), p53 degradation, and immortalization activity (41).

E6 and E7 genes have been studied in diverse populations, and different variants on these genes have been found associated with a higher risk of cancer in situ and invasive cervical cancer (14,37,48). Some authors have suggested that presence and persistence of

certain variants can increase the risk of cervical disease (12,19,28,44,49).

Radiation treatment is one of the most standardized and effective methods for cervical carcinoma therapy, and physiologic and cellular changes associated with high doses of irradiation have been documented (9,15,18).

However to date, little is known about the effect of ionizing radiation over the DNA of HPV and expression of E6/E7 oncoproteins (2,13,16,27,38), and there are not studies that analyze its effect in the apparition, presence, and persistence of HPV variants.

In Colombia, a country with high incidence of cervical cancer, there are not reports about the presence of E7/HPV16 variants and also there are not studies about the radiotherapy effect in the DNA viral and its variants in patients with cervical cancer.

The main goal of this study was to detect the presence of HPV and E7/HPV16 variants in sixty patients with cervical cancer (stages IIB and IIIB) before and three months after radiotherapy.

MATERIALS AND METHODS

Study population

Sixty (60) patients with invasive cervical cancer (stages IIB and IIIB) and aged 28-65, who attended the outpatient gynaecology service at the Instituto Nacional de Cancerología during 1995 in Bogotá, Colombia, were submitted to unique treatment with radiotherapy.

At recruitment, all women answered a questionnaire with clinical and general information; a database was built with this information and with data from their clinical histories. Informed consent was obtained from all study participants.

The Ethical Committee of the Instituto Nacional de Cancerologia approved the study protocol. After the interview, and before treatment, all women were asked to undergo to gynaecological examination to provide a cervical scrape (for cytological evaluation and HPV testing). Radiotherapy treatment was followed strictly for each patient and after three months of finished, the patient was called again to undergo to gynaecological examination and HPV testing.

Radiotherapy

All patients underwent radical radiotherapy as primary treatment of invasive cervical carcinoma. External-beam irradiation was delivered to the whole pelvic region and, if needed, to the para-aortic region depending on disease status. Total doses of external beam irradiation to the parametrium ranged from 4500 to 5040cGy (180-200 cGy every day). External-beam irradiation was followed by high-dose rate intracavitary brachytherapy with 3.500 cGy at point A (600cGy every day) and 1000cGy at point B.

Biological specimens

During gynaecological examination, cervical scrapes were collected from each woman by using two endocervical brushes. The first brush was used for routine Pap smear. The remaining cells of the first brush and the second brush were placed in a tube containing 5 ml of phosphate-buffered saline (PBS 1X) +0.05% thiomersal and processed according to Jacobs et al. (1995) (22). For analysis, 100µl aliquots were boiled for 10 minutes at 100ºC, cooled on ice, and centrifuged for 1 minute at 3,000 g. 10µl of this pre-treated crude cell suspension were used for PCR analysis. To asses the quality of the DNA target, a 209 base pair amplifying Beta-globin PCR was done by using the primer combination BPCO3 and BPCO5.

HPV detection and typing

HPV-DNA detection was performed through a standard GP5+/GP6+ PCR based assay, as described De Roda et al. (1995) (7). Briefly, forty cycles of amplification were carried out using a Perkin-Elmer 9600, USA thermocycler. Each cycle included a denaturation step at 94ºC for 1 minute, one annealing step at 40ºC for 2 minutes, and a chain elongation step at 72ºC for 1.5 minutes. The first step was preceded by a denaturation step of 4 minutes, and the last step was followed by an elongation step of 4 minutes.

Two dilutions of the cell line SiHa containing 1-10 copies of HPV16 (10 and 100 pg) and two dilutions of the cell line HeLa containing 10-100 copies of HPV18 (10-100 pg) were used as positive controls. Distilled water and processing blanks were used every tenth samples as negative PCR controls.

HPV positivity was assessed by non-radioactive southern blot hybridization of GP5+/GP6+ PCR products with a cocktail probe of specific digoxigenin-labeled DNA fragments from cloned DNA of HPV6, 11, 16, 18, 31, and 33, under low stringency conditions (1).

HPV positive samples were subjected to an enzyme immune assay (EIA) by using oligospecific probes to identify HPV types 16, 18, 31/33, 45 and, 52/58, according to Jacobs et al. (1997) (23).

E7/HPV-16 PCR amplification

The detection of E7/HPV16 was performed using a set of primers that amplify a fragment of 318bp (nt position 536-854). Specific primers, sense (5´-GGGGATCCGACACGTAGAG AAACCCAGCTGTAA-3´) and antisense (5´-GGCTGCAGGGTTTCTGAGAACAGATGGG GCACAC-3'), were used for PCR amplification. Briefly, the reaction was carried out in 50 µl of PCR solution containing 20 mM of Tris-HCl pH 8.3, 50 mM of KCl, 0.2 mM of each deoxynucleotide, 3.5 mM of $MgCl_2$, 1 U of DNA polymerase (Gibco BRL), 25 pmol of each primer, and 10 µl of sample. The PCR amplification consisted of DNA denaturation at 95ºC for 4 minutes, followed by 30 cycles of amplification using a PE 9600 thermocycler (Perkin-Elmer, USA). Each cycle included a denaturation step at 95ºC for 1 minute, one annealing step at 60ºC for 2 minutes, and a chain elongation step at 72ºC for 2 minutes. The final elongation step was extended for another 4 minutes.

The cell lines SiHa and Caski (10-100pg) were used as positive controls. The cell line HeLa (10-100 pg), distilled water and processing blanks were used as negative PCR controls.

SSCP analysis

The amplified PCR products were analysed by using a non-radioactive SSCP (single strain conformational polymorphism) (17) and by using 5% polyacrilamyde gels under no denaturant conditions (10% de glycerol, TBE 0.5X). 4µl PCR products were mixed with denaturant buffer (95% formamide, 20mM EDTA, 0.05% xylene cyanol, and 0.05% blue bromophenol). The samples were denatured at 95ºC and running at 4ºC and 300 V. Staining of the gels was done with 2% of $AgNO_3$. A densitometry analysis of the gels was done by using a Bio-rad GS-700. SSCP patterns from each specimen were compared individually with the reference patterns of prototype plasmid HPV16 (pHPV16) DNA, SiHa and Caski cellular DNA.

Direct sequence

In order to characterize nucleotide alterations of E7/HPV16, a subset of samples that showed different SSCP patterns were additionally analyzed through DNA direct sequencing. In brief, the amplified PCR products were purified with a DNA purification kit (Wizard PCR Prep., Promega) and sequenced by using a model 373 DNA sequencing system (Perkin Elmer, USA) and analyzed by using the Sequence Navigator software (Perkin Elmer, USA). Sequences were determined in both senses.

Statistical analysis

The size sample of this study was established by convenience. Contingence tables and t test for paired data were done to associate HPV presence and radiation effect. Only values of $p < 0.05$ were considered significant. Statistical analysis was done by using the SPSS 11.5 software (2006).

RESULTS

Characteristics of the study population are summarized in Table 1. Most of the women were aged 46-60 years (median age 50 years), had 4 or more parities (73.2%), reported a single life long sexual partner (51.7%) and had the first sexual intercourse between 16-19 years (44.1%). 53.3% of the women were classified in clinical stage IIIB and 65% had a tumor size >5cm.

Before radiotherapy, 50 patients (83.3%) were positive for generic HPV infection (Figure 1, A and B); 53.3% of them were positive for HPV16, 13.3% were positive for HPV31/33, 8.3% were infected with other HPV types, 5% were infected with HPV18, and 3.3% were positive for HPV45 or 52/58 (Table 2). Three months after treatment, 55 patients attended to control and 19 women (34.6%) were positive for generic HPV infection, 10.9% of them were positive for HPV16, 1.8% were positive for HPV18, 1.8% were positive for HPV31/33, and 20.2% were positive for others HPV types (7 of them (12.7%) with incident HPV infection (Table 2). The decrease of HPV detection after treatment was statistically significant ($p < 0.0005$).

From 32 HPV16 positive samples, 20 (62.5%) amplified to E7/HPV16 gene (Figure 2), 18 of them (90%) showed a SSCP pattern identical to the prototype, and 2 samples showed a different SSCP pattern (Figure 3, A and B).

The direct sequence of some samples that showed the same SSCP running pattern to the

Table 1. Characteristics of the study population

	No.	Overall %
Total	60	100
Age (years) +		
< 30	3	5.0
31-45	19	31.6
46-60	32	53.3
>60	5	8.3
missing	1	1.6
Age at 1st sexual Intercourse		
<15	22	36.6
16-19	26	43.3
20-23	8	13.3
>24	3	5.0
missing	1	1.6
Number of regular sexual partners		
1	30	50.0
2-5	23	38.3
>6	5	8.3
missing	2	3.3
Parity		
0	2	3.3
1-3	13	21.6
4-6	20	33.3
>6	21	35.0
missing	4	6.6
Clinical stage		
II B	28	46.6
III B	32	53.3
Tumor Size		
≤ 5 cm	19	31.6
> 5 cm	39	65.0
missing	2	3.3

prototype did not show presence of mutations, and the samples that presented a different SSCP pattern showed the presence of silent mutations at position 732 (T-to-C), position 789 (T-to-C), and position 795 (T-to-G). The same mutations were present in both samples (Figure 4).

After radiotherapy treatment, the presence of new changes (mutations) was not detected in any patient, and only 6 patients showed persistent HPV16 infection with a SSCP pattern similar to the prototype. In addition, the samples that initially showed a different SSCP pattern became negative to E7 HPV16 after radiotherapy.

DISCUSSION

This one is a pioneer study about the HPV infection and the presence of E7/HPV16 variants in women with cervical cancer before and after radiotherapy. We observed a high prevalence (83%) of HPV infections in women with cervical cancer before treatment with radiotherapy. These results are similar to others reported worldwide, where HPV is found in a prevalence range between 75% and 100%. These differences are mainly due to the detection systems employed and their sensitivity or due to sample inadequacy or integration events that affect the HPV L1 gene (45).

After radiotherapy treatment, a statistically significant decrease was observed in the detection of HPV at three months of follow-up. This decrease in the detection can have several explanations. The first one is the direct effect of the ionizing radiation over the HPV DNA, which alters its structure and affects the replication cycle. The second explanation could be that ionizing radiation affects all neoplastic cells, independently whether they are HPV infected or not. There is a study carried out by DeWeese et al, which shows that a neoplastic cell line (RKO) genetically modified to express the E6 and E7 oncoproteins, is equally sensitive to radiotherapy as no modified cells (8).The last explanation is a bad DNA quality or a low number of cells in the cervical scrapes took after radiotherapy. However, this explanation is less likely to occur due that all samples were positive for the amplification of the β-globine gene that provides information about the sample quality.

Table 2. HPV infection in patients with cervical cancer before and after radiotherapy treatmen

	Before Radiotherapy n=60 patients		After Radiotherapy n=55 patients	
HPV Types	No	%	No	%
Negative	10	16.6	36	65.4
Positive	50	83.3	19	34.6
16	32	53.3	6	10.9
18	3	5.0	1	1.8
31/33	8	13.3	1	1.8
45	1	1.6	--	
52/58	1	1.6	--	
Other types	5	8.3	11*	20.0

* 7 of the women with incident HPV infections.

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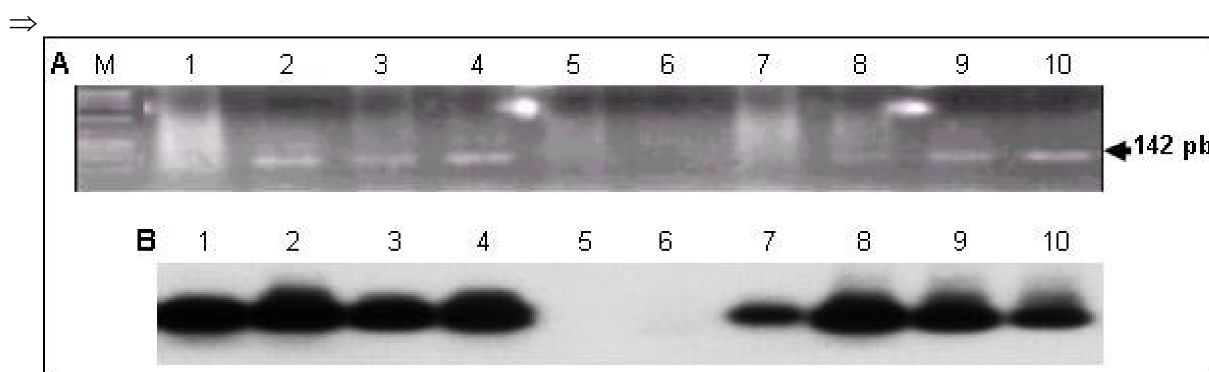

Figure 1. GP5+/6+ PCR and southern blot analysis of cervical scrapes of women with invasive cervical cancer before radiotherapy.
Lanes 1-5, 7-8: GP5+/6+ PCR products of cervical scrapes. Line 6: negative control (PCR mixture with water). Lanes 9-10: positive control (SiHa DNA 1ng and 100pg respectively).
(A) GP5+/6+ PCR products after gel electrophoresis.
(B) PCR products after southern blotting and hybridization with the HPV cocktail probe.

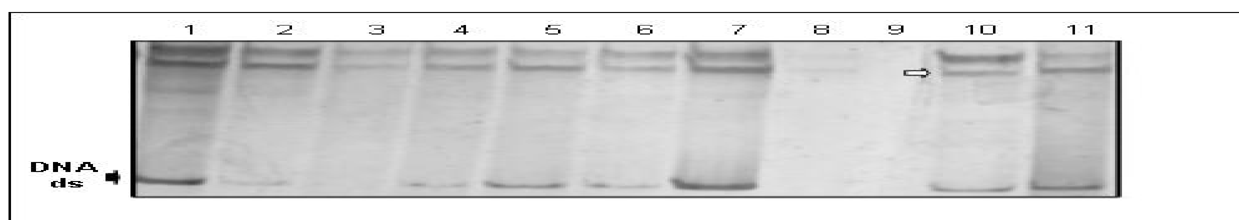

Figure 2. SSCP of E7/HPV16 PCR products of cervical scrapes of women with invasive cervical cancer before and after radiotherapy.
Lane 1: positive control, Caski DNA 1ng. Lane 2-8, 10-11: E7/HPV16 PCR products of cervical scrapes. Lane 9: negative control, Hela DNA 1ng.

The detection of HPV16 along time showed that six samples were positive for HPV throughout follow-up. In those cases, we could think that there was no radiotherapy effect on the DNA of HPV. A possible explanation is the presence of normal HPV infected cells continuous to the tumour that not received the direct effect of radiation. There are post-irradiation studies which show the presence of HPV after radiotherapy and/or its association with the developing of dysplasias and condylomes (2,13,29,35).

Likewise, we observed a higher prevalence and persistence of HPV16 infections compared with other HPV types, which confirms the major role of this type in the development of cervical cancer, results that are in agreement with other studies (20,32,34,46).

From 32 HPV16 positive patients only 20 amplified to E7 of HPV16. This difference in the detection could be due to efficiency of the PCR performed to amplify the E7/HPV16 gene compared to the efficiency of the PCR employed in the type-specific detection. The size of fragment amplified (318 bp versus 150 bp) could be a disadvantage in these kind of samples (42).

The SSCP analysis of the controls (pBR HPV16, Caski and SiHa) did not show running differences patterns. However, the direct sequence of SiHa cells showed a single mutation at position 645 (change of an adenine for a cytosine) that has been previously reported (4).

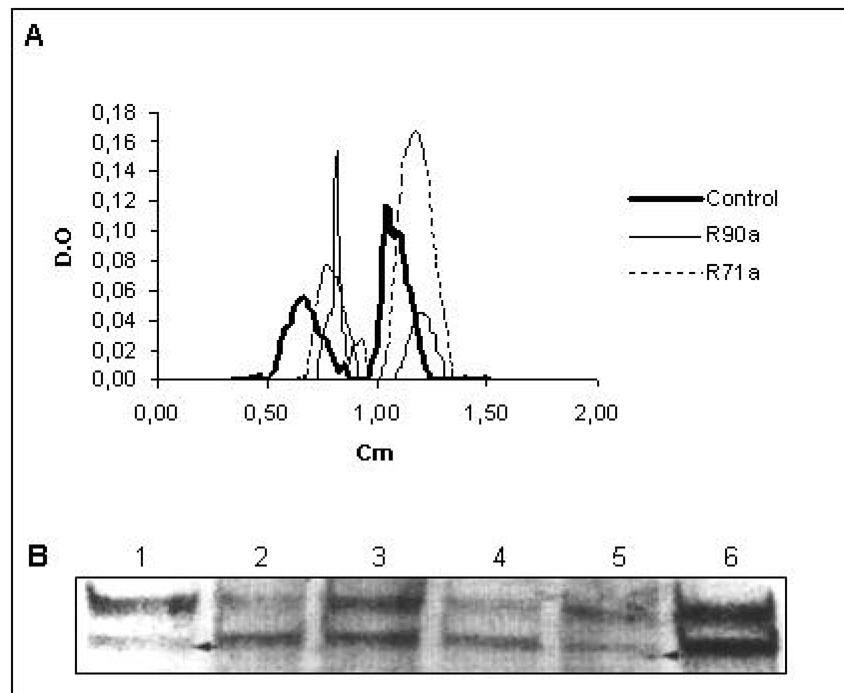

Figure 3. Densitometry analysis and SSCP of E7/HPV16 PCR products of samples that showed a SSCP different pattern.
(A) Densitometry analysis. R90a and R71a were samples that before radiotherapy showed a different pattern to the prototype. Control: Caski.
(B) SSCP of E7/HPV16 PCR products. Lane 1 and 5: samples with a different SSCP pattern to the prototype (R90a and R71a). Lane 2, 3, 4: samples with a similar SSCP pattern to the prototype. Lane 6: positive control Caski DNA 1ng

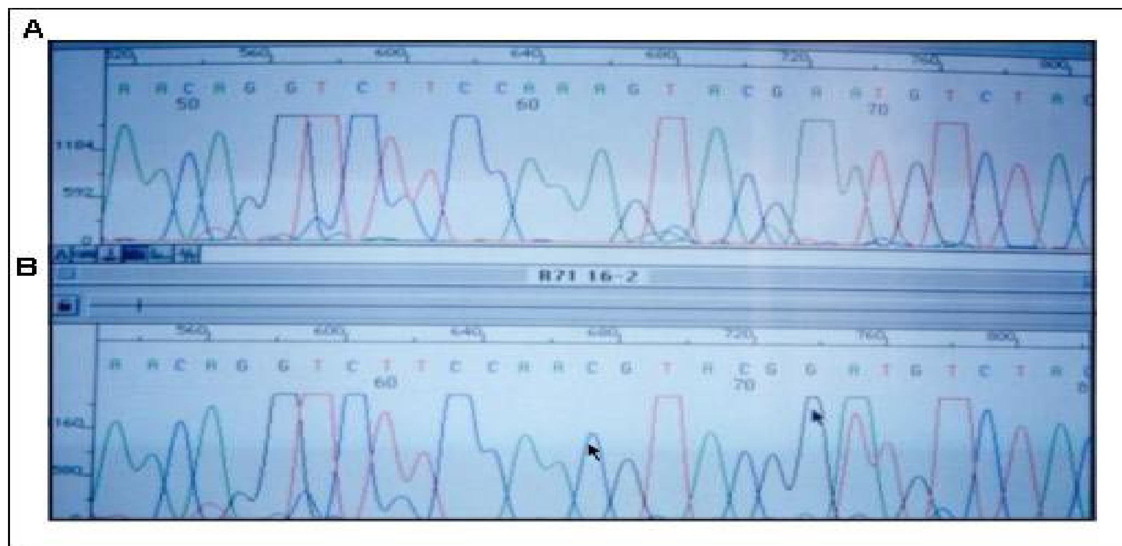

Figure 4. Direct sequence of a sample that showed though SSCP a different running pattern.
(A) Direct sequence of the prototype (Control, Caski, DNA).
(B) Direct sequence of the sample (R71a). Changes at position 789 and 795.

The SSCP analysis in the majority of the samples showed a similar running pattern to the controls (pBR HPV16 and Caski); these results are according with other studies that show a low variability in the E7 region of HPV16 genome in comparison with the reported for L1 and L2 regions and LCR (4,5,47).

The direct sequence of the samples that showed a different running by SSCP indicated the presence of silent point mutations at positions 732, 789 and 795. The mutations at nt 789 (T-C) and 795 (T-G) have been previously reported in patients with cervical cancer from non-European geographical regions (4,11,21,37,39,). In a study with Ugandan samples, Buonaguro et al (4) showed the linkage of both mutations (789 and 795) to specific mutation patterns of LCR, L1 and/or E5 and E6, and suggested that these changes are distinctive of the African lineage (Af1) (4). However, we do not have any information about the ethnic origin of the patients that carried these mutations in our study.

An aim of this study was to observe the effect of the ionizing radiation on the apparition and persistence of E7/HPV16 variants. Although the samples analyzed were few, the results showed that, after radiotherapy treatment, the occurrence of a new change (mutation) was not detected in any patient. We observed that six patients that were infected by HPV16 before radiotherapy showed persistence of HPV16 infection after radiotherapy and showed the same SSCP pattern to the controls pBR HPV16 and Caski. These results probably indicate that the ionizing radiation did not cause mutations in the E7 region. Another possibility is either that radiotherapy caused few or small mutations that were not predominant over variants already established or that the mutations occurred were effectively corrected by the DNA repair system.

Although our present study have some weakness, as the few number of samples studied and the short follow up, also have several strengths because until the moment there is few information concerning to the natural history of HPV infection in cervical carcinoma managed with radiation therapy.

The information that a prototype E7/ HPV16 variant predominate in Colombia, is very interesting in the knowledge of the variant distribution worldwide and confirms the reported in previous studies on E7 gene variants, which have suggested that the E7 oncoprotein is highly conserved in most populations (11,14, 21,37).

It is important to continue studying this region in view of the stability and low mutation rate of the E7 gene. The E7 protein can be used as a target of the immune response against the HPV infection, it can act as a specific tumour antigen, and it can active a cellular immune response, becoming in an attractive target for therapeutic immune intervention.

In conclusion, there was a decrease in the detection of HPV infection after three months of radiotherapy treatment and few E7/ HPV16 variants were detected, it seems that the treatment did not cause mutations in this gene. The details of the time course of HPV DNA variants clearance in invasive cervical carcinoma managed with radiation therapy remain to be elucidated by further studies.

ACKNOWLEDGEMENTS

This article is dedicated to Dr. Óscar Orozco who passed away during the development of this project. The authors are indebted to all the study participants, gynaecologists, radiotherapy group and nurses who collaborated in the fieldwork. Thanks are also to Dr. Héctor González, for helpful collaboration in the clinical part, and to Claudia Molina and Esperanza Trujillo, for technical support.

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