

Detection of Human Papillomavirus in Basal Cell Carcinoma by Polymerase Chain Reaction

Detecção do papilomavírus humano em carcinomas basocelulares por meio da reação em cadeia da polimerase

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Summary

The aim of the present study was to determine the presence of human papillomavirus (HPV) in tumors and sun-nonexposed normal skin from patients with basal cell carcinoma (BCC). Twenty-three patients with BCC had their tumors and axillary skin assayed for the presence of HPV by polymerase chain reaction (PCR), using degenerate primers of the L1 region. Controls were preliminarily established by using viral warts as positive control, and control axillary skin from healthy subjects as negative control. HPV was detected in 60.9% of the tumors and 43.5% of the patient's axillary skin. This difference was not statistically significant ($p > 0.05$).

(Villela Berbert ALC, De Oliveira Mantese SA, Goulart Filho LR, Rocha A, Machado Saraiva AC. Detection of Human Papillomavirus in Basal Cell Carcinoma by Polymerase Chain Reaction. *Med Cutan Iber Lat Am* 2004; 32(5): 205-209)

Key words: human papillomavirus, PCR, basal cell carcinoma.

Resumo

O objetivo deste estudo é determinar, nos pacientes portadores de carcinomas basocelulares (CBC), a presença do papilomavírus humano (HPV) nos referidos tumores, comparando-a com a pele sadia extraída de região fotoprottegida. Para tal, 23 pacientes com CBC tiveram o tumor e pele axilar estudados quanto à presença do HPV, através da reação em cadeia da polimerase, utilizando primers degenerados da região L1. Preliminarmente o controle da metodologia empregada foi estabelecido sobre verrugas vulgares, que reconhecidamente tinham a presença do HPV, confrontadas com tecido cutâneo de indivíduos sadios e presumidamente isentos de tal vírus. O HPV foi evidenciado em 60,9% dos tumores estudados, e em 43,5% da pele obtida das respectivas axilas dos pacientes, mas esta diferença não foi estatisticamente significativa ($p > 0,05$).

Palavras-chave: papilomavírus humano, PCR, carcinoma basocelular.

Basal cell carcinoma (BCC) is the most common malignant skin tumor in humans, accounting for 71% to 75% of nonmelanoma skin cancers[1, 2]. Its incidence has increased worldwide, particularly in tropical countries. In parallel, an increasing development of scientific researches has shown a strong association of human papillomavirus (HPV) with cervical cancer and cutaneous squamous cell

carcinomas (SCC)[3-5]. Studies showing this association have been well documented due to recent improvement in molecular biology techniques, making possible not only a more accurate confirmation of the presence of HPV in lesions but also viral typing through recognition of new HPV types, correlating them to benign and malignant skin lesions[6-8].

Polymerase chain reaction (PCR) can demonstrate HPV DNA even in paraffin-embedded material or when only small amounts of samples are obtained[9, 10].

Only a few studies have investigated the possible role of HPV in the etiology of BCCs, and the results seem to be conflicting among the different assays used. Eliezri et al. (1990)[11] investigated the presence of HPV in 94 lesions of 89 patients with either SCC (68 lesions) or BCC (26 lesions), using in situ hybridization or hybridization with filter, and detected HPV DNA in 36 (52.9%) of SCC and in only one (3.8%) BCC. Tumors located in the genital area and fingers showed HPV DNA in 68% and 75%, respectively, most of them containing HPV16 or related sequences. The authors suggested that HPV is not associated with SCC occurring in other sites, except for genital area and fingers, emphasizing that such evidence is not applicable for epidermodysplasia verruciformis cases.

Pierceall et al. (1991)[12] studied 21 SCC and 17 BCC cases by PCR using specific primers for HPV16 and HPV18 to amplify DNA in regions E6-E7 and found positivity in 19% in both groups.

Saegusa et al. (1995)[13] assayed 12 BCC of the eyelid by PCR in material previously fixed in 10% formol and embedded in paraffin, and all specimens were negative for HPV DNA.

Shamanin et al. (1996)[1] studied nonmelanoma skin tumors in a group of immunosuppressed individuals submitted to renal transplant compared to a control group of immunocompetent subjects. DNA of each sample was amplified using 16 combined primers, thus enhancing the sensitivity and specificity of PCR. HPV was detected in 65% of 20 SCC and in 60% of 5 BCC in the immunosuppressed individuals, while in the immunocompetent group, HPV was found in 31% of 26 SCC and 36% of 11 BCC cases.

Boyd et al. (1996)[14] pointed out the possibility of obtaining false-positive results in the detection of HPV by PCR. The probable involved factors include the contamination of nucleic acid through material or people working there, saliva, hair or keratinocytes. False-negative results can be obtained from errors in the mixture of the reaction, failure in the reagents, cycles at inadequate temperatures, insufficient DNA samples, and the low quality of DNA obtained through paraffin-embedded tissues, as previously mentioned.

De Villiers et al. (1997)[15] used two extensive combinations of primers for PCR and obtained surprising results in relation to positivity for sequences of HPV DNA. However, only two BCC were studied, with negative results.

Astori et al. (1998)[16], using two different primers, one containing 16 different combinations as described by Shamanin et al. (1994)[17] and another with two additional combinations, did not find sequences of HPV DNA in 3 BCC

studied. In perilesional skin obtained from patients with sebaceous epithelioma, keratosis verrucosa, actinic keratosis, BCC and two intra-epithelial carcinomas, HPV was found in 50% (3 samples) of both the lesional and perilesional areas of actinic keratosis, and only in the perilesional area of keratosis verrucosa and sebaceous epithelioma. The authors pointed out the presence of HPV DNA in 35% of normal skin studied, suggesting that the virus might be widely distributed on the skin of immunocompetent people, whose immune system would prevent the development of disease. Additionally, they emphasized the role of ultraviolet radiation in the induction of HPV genes or mutational inactivation of cellular genes that usually suppress the activity of HPV genes.

In the present study, we evaluated patients with BCC for the presence of HPV in tumors and normal skin derived from a sun-nonexposed area (axilla) by PCR. The confirmed cases were examined in relation to gender, age, skin type, tumor area, duration and site of the lesion.

Patients and Methods

Patients

We studied 23 patients with BCC attending the Hospital das Clínicas, Universidade Federal de Uberlândia (HC/UFU), MG, Brazil, from May to December 1998, for the presence of HPV. The tumors were diagnosed in the Dermatology Ambulatory (HC/UFU) and classified into the following clinic types: nodular, nodule-ulcerated, superficial, rodent ulcer, vegetans, pigmented, sclerosing, cicatricial, and cystic. None of the patients had a history of immunosuppression or multiple skin malignancies starting at precocious age, previous or actual radiotherapy/chemotherapy, and the skin tumors were not under treatment when excised. Skin samples from 5 patients with common warts (positive control) and 9 healthy subjects (negative control) were used as controls.

All patients were submitted to a questionnaire on personal information, sun-exposure and skin phototype. An informed consent was obtained from each patient.

Dermatological procedures

In patients with more than one tumor, preference was given to those located on the face, selecting randomly only one tumor in this site in each patient for statistical proposals. After surgical excision, a 4 mm central fragment was removed by punch and immediately stored at -70°C for further assay by PCR. The remaining tumor was fixed in a 10% formol solution for histopathological analysis. Common warts (positive controls) were removed by shaving, bisected, and subjected to the same procedure.

In parallel, a skin sample of the right axillary area, with no clinical changes, was removed by punch after xylocaine

anesthesia, as described by Stark et al. (1994)[23]. The sample was bisected and also sent for histopathology and PCR. Similarly, normal skin fragments (negative controls) from 9 healthy patients were obtained from plastic surgeries (5 blepharoplasties, 2 mammoplasties and 2 abdominoplasties).

PCR for HPV DNA

All previously frozen tissue samples were macerated in liquid nitrogen, following thawing and homogenization in 600 µl of sample buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1mM EDTA, 1% sodium dodecyl sulfate, and 500 µg/ml proteinase K) and incubation at 55°C overnight. After digestion, the samples were extracted by adding 200 µl of saturated NaCl buffer, centrifuged at 25,000 g for 15 min at 4°C. The supernatant was saved in another new microtube and equal volume of isopropanol was added. The DNA pellet was precipitated by centrifugation at 25,000 g for 15 min at 4°C, washed in 70% ethanol, dried and resuspended in sterile water.

The DNA samples were amplified by PCR, using the primers within the L1 open reading frame of the HPV genome: MY11 (5'GCMCAGGGWCATAAYAATGG 3') and MY09 (5' CGTCCMARRGGAWACTGATC 3') where M=A+C, R=A+G, W=A+T and Y=C+T (TING & MANOS, 1990)[19]. The amplification step was performed using a MJ Research Thermocycler (Molecular Genetics Laboratory-UFU, Uberlândia, Brazil) as follows: 36 cycles, 95°C for 30 s (denaturation), 55°C for 40 s (annealing), and 72°C for 60 s (extension). PCR products were submitted to 1.5% agarose gel electrophoresis stained with ethidium bromide, observed and recorded at the ImageMaster Video Documentation System (Pharmacia).

Statistical analysis

Data were statistically analyzed using the Biological Research GMC software, version 7.3. PCR results obtained for positive (warts) and negative (normal skin from healthy subjects) controls were analyzed by the Fisher exact test. The correlation between variables was analyzed by the Pearson correlation coefficient. The following scores were arbitrarily determined for PCR results: (0) negative tumor and axilla; (1) negative tumor and positive axilla; (2) positive tumor and negative axilla; (3) positive tumor and axilla. A P value <0.05 was considered as statistically significant.

Results

The present paper reports a cross-sectional study in patients with BCC diagnosed by dermatological exams. These patients were investigated for the presence of HPV in

Table 1. PCR results and characteristics of the tissue specimens analyzed in 23 patients with BCC.

Patient	Site	Tumor size (cm ²)	Tumor age (month)	PCR
1	Face	1.95	24	+
	Axilla			-
2	LLL	42.00	48	-
	Axilla			-
3	Face	1.21	24	-
	Axilla			+
4	Face	2.25	27	-
	Axilla			-
5	Face	1.44	24	-
	Axilla			-
6	Face	1.20	18	+
	Axilla			-
7	Face	3.00	30	+
	Axilla			-
8	Face	1.20	36	+
	Axilla			+
9	Thorax	3.00	24§	+
	Axilla			+
10	Neck	2.04	12	-
	Axilla			+
11	Face	3.00	60	+
	Axilla			+
12	Face	0.35	24	-
	Axilla			-
13	Face	0.42	7	+
	Axilla			+
14	Face	0.64	8	+
	Axilla			-
15	Face	4.20	30§	+
	Axilla			+
16	Face	3.00	9	+
	Axilla			+
17	Face	2.70	12	+
	Axilla			+
18	Face	0.77	12	-
	Axilla			+
19	Neck	1.95	6	+
	Axilla			-
20	Face	1.08	8	-
	Axilla			-
21	Face	0.70	6	-
	Axilla			-
22	Face	1.90	5§	+
	Axilla			-
23	Face	2.24	6	+
	Axilla			-

tumors and axillary skin. Among the 23 studied patients, 12 were female (52.2%) and 11 male (47.8%), with age ranging from 34 to 87 years (65.65 ± 14.96 yr). The skin types were classified in phototypes from I to VI, according to Melski et al. (1977)[20]. Thus, skin type I (always burns, never tans) was found in 10 subjects (43.5%) and type II (usually burns, sometimes tans) was also seen in 10 subjects (43.5%). Only 3 subjects (13%) had skin type III (sometimes burns, usually tans). No case of skin types IV to VI was found.

All the warts used as positive controls were positive for HPV DNA by PCR, as determined by the fragment size of 450 bp. On the other hand, PCR results were consistently negative in all samples from healthy subjects that served as negative controls (data not shown). Therefore, PCR results obtained from warts (positive control) were significantly different ($p < 0.0001$) when compared to those obtained from skin fragments from healthy subjects (negative control), thus evidencing a high accuracy in the assay for the detection of HPV.

As demonstrated in Table 1, out of 23 patients with BCC, PCR results showed HPV DNA sequences in 14 (60.9%) tumor samples. On the other hand, normal axillary skin showed positive PCR results in 10 (43.5%) of 23 patients. Two samples also revealed two distinct bp sizes, but were not obtained from the same patients that showed double bands in the tumors. The frequencies of positive PCR results for HPV in the tumor were not significantly different ($p > 0.05$) from those found in the axilla from all 23 studied patients. However, these results were independent, that is, a positive HPV result in the tumor was not necessarily associated with a positive HPV reaction in the axillary skin from the same patient and vice-versa. Similar results were obtained from the tumors located in the face, where the analysis of data in this region was separated because it is a site more exposed to sun, probably favoring the appearance of tumors.

An attempt was made in order to seek associations between PCR results related to the axilla and personal data of patients or tumor characteristics, but no association was found to be statistically significant ($p > 0.05$) by the Fisher test.

Comment

Ito & Evans (1961)[21] demonstrated that the HPV genome acts as a single carcinogen, inducing the appearance of carcinomas in rabbits by inoculation of viral DNA. Pierceall et al. (1991)[12] reported positivity for HPV in three of 16 BCC cases obtained from sun-exposed sites, and no

positivity was found in cases from nonexposed healthy skin. Similar results were obtained in 21 SCC (four positive cases). The authors suggested that HPV16 would be involved in the pathogenesis of some skin tumors induced by ultraviolet radiation[22]. In spite of the great predominance of tumors located in the face, in the present study, statistically significant differences were not found between face tumors and those located in areas less exposed to the sun. These results might indicate that patients with BCC can harbor HPV in healthy and nonexposed skin sites. Various HPV types have been found in healthy skin[16, 18], suggesting that HPV DNA can be widely distributed in the normal skin of immunocompetent people. It is still unclear whether a specific molecular mechanism of the effective action of HPV on the carcinogenesis or an interaction of HPV with other external factors, such as ultraviolet radiation[22], association with herpes virus or chemical carcinogens such as tobacco or arsenic, would favor this sequence of events[23].

Another important question to be investigated is whether the HPV virus is found in the skin only as a contaminant particle derived from the environment (contact) or the virus has invaded the cell and incorporated into the genome, becoming a tumor factor. In this case, PCR assay detects only the presence of the virus, without demonstrating its cellular invasion, which is usually performed by *in situ* hybridization or *in situ* PCR. If were only by contact, the detection would be erroneous with positive and negative results in samples from a single patient, as demonstrated in both the tumor and the normal skin from some patients. However, the non-detection of HPV in healthy subjects can suggest a possible role for the virus in the development of the tumor, associated with other environmental and genetic factors[23].

Taken together, it can be concluded that the presence of HPV in the tumors (60.9%) and the axillary skin (43.5%) from patients with BCC was high, although this difference was not statistically significant. In addition, PCR results in tumors and axillary skin showed to be independent factors, that is, a positive reaction for the tumor was not necessarily associated with a positive reaction for the axilla and vice-versa. Nevertheless, these findings suggest that HPV might be disseminated only in patients with tumors, since it was not found in healthy subjects. The presence of HPV in approximately 74% of the patients with BCC, independently of the assayed tissue, allied to non-detection of HPV in healthy subjects could suggest a possible role for the virus, associated with environmental and genetic factors, in the etiology and pathogenesis of BCC.

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