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α-HPV positivity analysis in a group of patients with melanoma and non-melanoma skin cancers

Analiza pozitivităţii α-HPV la un grup de pacienţi cu tumori cutanate melanocitare şi non-melanocitare

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Abstract

Background. *Medical research has shown a continuous increase in the incidence of skin cancers, especially among young individuals. One of the ethiopathogenic factors that cause skin carcinogenesis could be the infection with some genotypes of human papillomavirus (HPV).* ***Methods.*** *In our study, we have analyzed alpha (α) - HPV positivity and HPV genotypes identified in melanocytic (MSC) and nonmelanocytic skin cancers (NMSC). The results were then compared with results obtained from the control group. The study included 40 cases of MSC and NMSC found in the data base of our hospital, and 40 healthy patients. In all of the cases, we identified the HPV DNA by using polymerase chain reaction (PCR), and the viral genotypes by using α –HPV primers by Linear Array Roche kit.* ***Results.*** *The average α-HPV positivity in tumors was 32.50%, higher than in other studies published to date. The squamous cell carcinoma (SCC) lot had the highest α-HPV positivity (40%), followed by basal cell carcinoma (BCC) (35%) and malignant melanoma (MM) (20%). The comparative analysis between skin cancer-HPV positive (32.50%) and the control group-HPV positive (15%) revealed a positivity of HPV in the tumors group (32.50%) that was higher by a ratio of 2.16. By viral genotyping, we identified high-risk HPV only in BCC and MM (in all α-HPV samples), but not in SCC samples.* ***Conclusions.*** *In our study, α-HPV in NMSC and MSC was positive in 32.50% of the cases; in 46.15% of these, it was possible to identify HPV genotypes. The high-risk HPV genotypes observed in these patients were HPV 16, 35, 58 and 59.*

Keywords*: human papillomaviruses, basal cell carcinoma, squamous cell carcinoma, malignant melanoma.*

Rezumat

Introducere. *În ultimele decenii, pe plan naţional şi mondial se observă o creştere continuă a incidenței tumorilor cutanate, cu apariția acestora la vârste tot mai tinere. Unul dintre factorii etiopatogenici carcinogeni implicați în procesul de carcinogeneză cutanată ar putea fi infecţia cu anumite genotipuri de papilomavirusuri*

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umane (HPV). ***Scopul cercetării.*** *În studiul nostru am analizat pozitivitatea α -HPV şi genotipurile HPV în tumorile melanocitare şi non-melanocitare. Rezultatele obţinute au fost comparate cu pozitivitatea α-HPV la lotul martor.* ***Material şi metodă.*** *În studiu nostru au fost incluse 40 de cazuri de tumori cutanate melanocitare (TCM) şi non-melanocitare (TCNM) aflate în evidența oncologică a spitalului nostru şi 40 de pacienţi sănătoşi. În toate cazurile am realizat identificarea acidului dezoxiribonucleic (ADN) al HPV prin PCR şi genotiparea virală utilizând primeri α–HPV, cu kitul Linear Array Roche.* ***Rezultate.*** *Pozitivitatea medie a α-HPV la nivel tumoral a fost de 32.50%, fiind superioară studiilor publicate până în prezent. Din lotul tumoral, carcinoamele spinocelulare (CSC) au avut pozitivitatea α-HPV cea mai mare (40%), fiind urmate de carcinoame bazocelulare (CBC) (35%) şi melanoame maligne (MM) (20%). Analiza comparativă a tumorilor cutanate HPV pozitive cu lotul martor HPV pozitiv a relevat o pozitivitate mai mare a tumorilor HPV pozitive (32.50%) comparativ cu lotul martor – HPV pozitiv (15%), raportul fiind de 2.16. Prin genotipare virală am identificat HPV cu risc oncogen înalt în CBC şi MM (în toate probele α-HPV pozitive), dar nu şi în probele de CSC.* ***Concluzii.*** *În studiul nostru, pozitivitatea α-HPV în TCNM şi TCM a fost de 32.50% iar identificarea genotipurilor HPV a fost posibilă la 46.15% dintre acestea. Genotipurile HPV cu risc oncogen înalt identificate la aceşti pacienţi au fost HPV 16, 35, 58 şi 59.*

Cuvinte cheie*: papilomavirusurile umane, carcinom bazocelular, carcinom spinocelular, melanom malign.*

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Introduction

The continuous increase in skin tumor incidence in patients of young ages calls for a better understanding of the carcinogenic factors involved in the development of these tumors. One of the factors contributing to skin carcinogenesis could be the infection with some genotypes of human papillomaviruses (HPV). Existing data are not very accurate and cannot determine their precise role in skin cancers. To date, there exist conflicting results regarding the genotypes of HPV (α-HPV or β-HPV) that may have a role in skin carcinogenesis (1,2).

The aim of this study is to investigate and analyse the α-HPV positivity in melanoma (MSC) and non-melanoma skin cancers (NMSC), the achievement of a qualitative quantification of α-HPV genotypes in MSC and NMSC (high-risk or low-risk HPV genotypes) and the HPV-positivity from MSC and NMSC in comparison to the control group.

Materials and Methods

40 patients with MSC and NMSC and 40 healthy skin patients (hereafter referred to as the 'control group') were genotyped and investigated for HPV positivity, after having given their informed consent.

The inclusion criteria were as follows: patients aged 18 years and older, the clinical diagnosis of MSC and NMSC with histopathological confirmation and immunocompetent patients.

The study was approved by the Ethics Committee of our Clinical Hospital.

For the processing of data we designed a standardised form based on the medical records of hospitalized patients, the registration of cancers from the Departments of Dermatology and Oncology, oncological records (from our hospital), histopathological records containing types of skin cancer and other genotyping records.

In our lot of patients with MSC (10 patients) and NMSC (20 patients with BCC and 10 patients with SCC) we identified α-HPV using polymerase chain reaction (PCR) from the tumoral samples. The α-HPV positivity were: 35% of BCC was HPV positive, 40% of SCC was HPV positive and 20% of MM was HPV positive. Genotyping was performed in collaboration with the Institute of Virology.

A demographically similar group to that with NMSC and MSC (in terms of age and sex) was formed as a control group, and all patients expressed their informed consent for the study. The control group was analysed using the same methods as the NMSC and MSC group, including the genotyping of HPV.

We chose to identify α-HPV due to their high aggressiveness in comparison to other HPV types (β-HPV are the most commonly identified types in skin tissues and therefore also the most investigated in medical research).

For statistical data processing we used the Statistical Program for Social Science (SPSS) version 10.

Biological samples

Tumor specimens were obtained by excision and/or by biopsy to be examined for the histopathological diagnosis. The biological material for viral testing consisted of 50 μm slices of paraffin wax embedded tissue (obtained from 40 patients with MSC and NMSC and 40 healthy patients from the control group).

DNA isolation

DNA extraction from *paraffin* wax embedded tissue consisted in xylene/ethanol *dewaxing*, followed by a kit based isolation. Briefly, the sections were dewaxed with two xylene washes (20 minutes at 37°C each) followed by two 100% ethanol washes. The rehydration was performed with 80%, 60%, 40% ethanol and distilled water washes. The isolation of total DNA was performed with High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's recommendations. Isolated DNAs were subsequently stored at 20°C. The concentration and purity of each DNA sample was evaluated with a NanoDrop spectrophotometer. The target DNA's integrity was verified by amplification of a 110 bp b-globin gene fragment using PC 03/04 primers synthesized by Invitrogen according to Saiki et al (3) (5'- ACACAACTGTGTTCACTAGC-3' and 5'-CAACTTCATCCACGTTCACC-3', respectively).

Detection of α-HPV

DNA detection was performed by means of nested PCR, according to Fuessel et al (4).

Degenerated primers MY09
(5'- CGTCCMARRGGAWACTGATC-3')
and MY11
(5'-GCMCAGGGWCATAAYAATGG-3')
designed by Manos et al (5), were used to amplify a 450 bp fragment from a L1 HPV gene in a final volume of 50 microliters of the reaction mixture containing 1x PCR buffer, 200 μM dNTP, 2mM $MgCl_2$, 2 U Taq DNA Polymerase and 100nM of each primer. Five microliter amplicons of the first reaction were subjected to nested PCR GP5 +
(5' ,- TTTGTTACTGTGGTAGATACTAC - 3') / 6+
((5',-GAAAAA–TAAACTGTAAATCATATTC -3')
in 50 microliters of the reaction mixture containing 1x PCR buffer (Promega), 200 μM dNTPs (Promega), 3mM MgCl2 (Promega), 2 U Taq DNA Polymerase (Promega) and 100 nM of each primer (sinthetyzed by Invitrogen). GP+ primers were designed by de Roda Husman et al (6).

As a positive PCR control, DNA isolated from CaSki cells was used while as negative control RNase free water was used instead of DNA target.

HPV DNA detection by PCR with FAP 59/64

These primers
(5'-TAACWGTIGGICAYCCWTATT-3' and 5'-CCWATATCWVHCATITCI-CCATC-3') target a region of about 480 nucleotides in the L1 ORF. The FAP59/64 PCR system is usually used for skin samples. PCR mix: 0.75 μM of each primer (Invitrogen), Fermentas master-mix PCR and target DNA.

10 microliters of each amplicon from each PCR were analyzed in 2% electroforesis gel following ethidium bromide staining.

HPV DNA genotyping

α-HPV-positive samples were genotyped using the LinearArray HPV Genotyping Test (Roche) according to the manufacturer's instructions. The biotinylated primers define a sequence of nucleotides within the polymorphic L1 region of the HPV genome of approximately 450 base pair long. The pool of HPV primers amplify HPV DNA from 37 human papillomavirus genotypes (including 13 high-risk genotypes (**16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)**, while an additional primer pair targets the human β-globin gene to provide a control for cell adequacy, extraction and amplification. Amplification was done as nested PCR, and HPV genotyping was performed according to the manufacturer's instructions. Since the primers used were biotinylated, the formed hybrids were detected using Streptavidin conjugated with alkaline phosphatase. Adding the substrate allowed us to visualize the immobilized hybrids. The high sensitivity (93%) and specificity (93%) recommended this kit for genotyping. Positive controls provided by the kit were used in order to validate the method.

Results

Upon analyzing the results we found that 32.50% from the genotyped tumors were α-HPV-positive (n=13 cases).

The distribution of this percentage on tumor types revealed that the highest number of α-HPV-positive tumors was encountered among SCC (40%), followed by BCC (35%) and MM (20%) (Table 1).

The DNA α-HPV detection was performed by PCR, using MY09/MY11 and GP+5/GP+6 primers (6 samples of BCC, 3 samples of SCC, 2 samples of MM and 4 samples from the control group were α-HPV-positive). Using FAP59/64 primers allowed us to detect 4 additional posi-

Table 1. Distribution of α-HPV positivity in our cases with BCC, SCC and MM

α-HPV positivity	Total number n (%)	BCC n (%)	SCC n (%)	MM n (%)
α-HPV +	13 (32.50%)	7 (35%)	4 (40%)	2 (20%)
α-HPV -	27 (67.50%)	13 (65%)	6 (60%)	8 (80%)

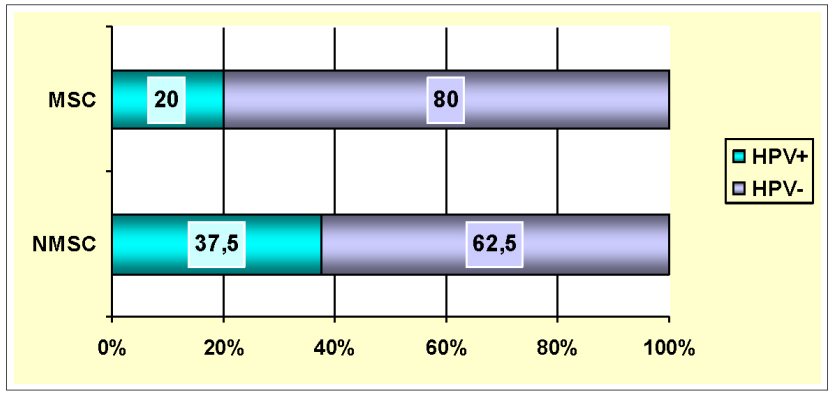

Figure 1. The report of NMSC-HPV+ / MSC-HPV+ in our group of patients.

tive samples (1 sample of BCC, 1 sample of SCC and 2 samples from the control group).

The ratio NMSC-HPV+ (37.50%) / MSC-HPV+ (20%) was supernumerary (1.86) (Fig. 1).

A comparative analysis of tumors-HPV+ with the control group-HPV+ showed a higher positivity of tumors-HPV+ (32.50% compared to 15% for the control group) with a ratio of 2.16.

The viral genotyping with Roche Linear Array kit identified a much smaller number of HPV genotypes (46.15% of all HPV+ samples).

From all the genotypes identified, 83.33% were "high-risk" oncogenic genotypes (HPV 16, 18, 35, 58, 59).

In BCC patients, the α-HPV genotypes were identified in 42.86% of the cases, all of which were "high-risk" (HPV 16, 58 and 59).

The HPV genotype identified in SCC (25%) was HPV 42, which is not a high-risk genotype.

In the MM group we identified the α-HPV "high-risk" (HPV 16 and 35) genotypes in both HPV positive cases.

All the identified HPV genotypes in BCC and MM were high-risk genotypes.

Within the control group the identified genotypes were "high-risk" (HPV 18) and "low-risk" (HPV 11).

Discussions

The oncogenic role of HPV in cervical cancer is well established; however in other cancers, it remains somewhat unclear. The malignancies in which HPV plays a significant role, either in favoring or determining carcinogenesis, are acral SCC, lung carcinoma, larynx, sinuses, conjunctival, esophageal, oral, vulvar, cervical (7), anal and penile cancers (8). The list of malignancies in which HPV is present has not yet been completed.

The identification of HPV in NMSC was the study subject of many papers, but the results of these studies could not clarify the exact role of HPV in skin carcinogenesis. HPV positivity in NMSC, MSC and healthy skin varies according to the primers and the technique used. The increasingly broad application of HPV DNA detection by PCR has determined the increase of HPV presence in NMSC, the most common being β-HPV. α-HPV genotypes that have sparked the interest of researchers were "high-risk" types (HPV 16, 18, 31, 33, 35, 39, 45, 50, 51, 53, 55, 56, 58, 59, 64, 68) associated with the development of anogenital carcinomas and dysplastic lesions. The HPV 16 is considered to have the highest risk in developing anogenital cancers (8).

In contrast to the HPV genotypes identified in the mucosa (α – HPV), HPV genotypes identified in skin tissues predominantly come from β – HPV types.

Modern techniques for identifying viral DNA have revealed a low prevalence of α – HPV in the skin (1). In some patients with HPV positive oncologic cervical lesions, HPV have also been identified in the skin of the hands (9). In these cases, the possibility of transmission of HPV from genital lesions was taken into account.

The necessity of using advanced techniques to identify the virus lies in the observation that only a few copies of HPV are present in skin cancers. Quantitative detection of HPV showed a variable number of HPV copies, ranging from one HPV copy in 14200 cells to 50 HPV copies in 1 cell (1).

In our group of patients (40 immunocompetent patients with NMSC and MSC), we have achieved viral genotyping using α-HPV primers. We obtained an α-HPV positivity of 32.50%. The percentage registered in our study was higher than the ones reported in similar studies (the α-HPV positivity in skin cancers is 10%) (1). These differences may be explained by the number of patients enrolled in our randomized study.

Regarding HPV positivity and the types of skin cancer, our study revealed that the highest number of α-HPV-positive tumors were SCC (40%), followed by BCC (35%) and MM (20%).

In previous studies, β-HPV positivity in tumor biopsies with BCC ranged from 0% to 44%

(7-9). In all reported studies, the prevalence of β-HPV was higher than that of α – HPV (4%) (1).

It is generally acknowledged that the rate of α-HPV positivity is lower than β-HPV positivity. Similarly, other studies showed an α-HPV positivity in BCC tissues that ranged between 0% and 18.8% (1). All of them had used PCR for detecting HPV.

The results of our study have revealed higher percentages of α-HPV positivity in BCC (7 of 20 BCC were HPV positive - 35%) compared to the above mentioned studies (with a larger group of patients) also using PCR as a method for determining HPV genotypes.

Studies that researched the presence of HPV in SCC showed a β-HPV prevalence that varied between 0 and 58.3% (1, 12). The α-HPV positivity showed variations between 0% and 30% (1, 13-15). All studies used PCR as a method for determining HPV positivity. Within a study conducted in 2006 on 247 patients with SCC, actinic keratoses and healthy control groups obtained a positivity of HPV 16 of 11% in the SCC (16).

In a study on 292 patients with SCC of cephalic extremity, the subtype 16 of α-HPV positivity was almost twice as high as in the control group (12% compared to 7%). The HPV 18, 33 and 73 had a relatively equal positivity in SCC and in the control group. A SCC labial biopsy releaved a rate of 4% in HPV 16 positivity (17).

In a study on 271 oropharyngeal cancers, HPV status was determined by PCR. The results showed that the increased incidence of oropharyngeal cancers in US is caused by HPV (18).

In our study, the α-HPV positivity in SCC was 40% (4 of 10 SCC were HPV positive). Only in one patient we identified the viral genotype HPV 42, which is part of low-risk HPV.

In some studies, HPV was identified in fragments of melanoma.

A study conducted in 2005 on 28 Greek patients with MM, regarding the presence of HPV 6, 11, 16 and 18 in tumor fragments, identified a positivity of 17.85%, which is comparable to the result of our study (20 %) (19).

Other studies found β-HPV positivity in MM tissues ranging between 2.78% and 13% (1).

A study conducted by Ruer in 2009 on the identification of α-HPV in the MM fragments identified an α-HPV positivity of 10.6%, predominantly of the HPV 16 subtype (2).

In our group of patients with MM, the α-HPV positivity was 20% and unveiled higher percentages than those found in literature, in other report to a larger group of patients (1). The isolated high-risk genotypes were HPV 16 and 35.

In 2009, Zaravinos et al conducted a study about viral HPV DNA detection in NMSC and found that 5 out of 15 BCC were HPV positive (33%), 4 of 12 SCC were HPV positive (33%) and 8 of 53 normal skin tissues were HPV positive (15%); the data is similar to the results of our study (20). Moreover, in this study the proportion of subjects that tested positive for α-HPV is relatively similar to that identified in our groups (35% vs. 33% in BCC; 40% vs. 33% in SCC specimens and 15% vs. 15% in normal skin tissue).

The literature also shows an HPV positivity ratio between NMSC and MSC of about 1. In our study, the HPV positivity ratio NMSC: MSC was 1.86 (NMSC-HPV positive – 37.5%; MM-HPV positive - 20%).

Our control group of 40 patients presented an HPV positivity of 15%. A comparative analysis between the NMSC and MSC group and the healthy control group revealed that HPV genotypes identified (32.50%) were 2.16 times higher in the tumor group than in the control group (15%).

SCC had an α-HPV positivity (40%) that was 2.67 times higher than in the control group

(15%); similarly, BCC and MM had a positivity that was 2.33 times and 1.33 higher than in the control group. This result supports the idea that HPV is actively involved in skin carcinogenesis.

Through viral genotyping, we identified a much smaller number of α-HPV genotypes (46.15% from α-HPV positive samples). Out of the isolated genotypes, 83.33% were "high-risk" oncogenic genotypes (HPV 16, 18, 35, 58, 59).

In patients with BCC, HPV genotypes were identified in 42.86% of the α-HPV positive samples, all of which were high-risk (HPV 16, 58 and 59). HPV genotypes that were identified in the SCC group (25%) were HPV 42, not included in high-risk HPV. In MM α-HPV positive patients, we obtained viral genotypes (HPV 16 and 35) in both HPV positive cases (100%).

Within the control group, HPV genotypes were identified in 33.33% of the α-HPV positive cases, of which 50% were high-risk and 50% were low-risk. A comparative analysis of the tumor group (66.66% of α-HPV genotypes have been identified with high-risk genotypes) and the control group (50% with high-risk genotypes) reveals a higher incidence of high-risk HPV genotypes in the tumor group, particularly in the group of MM (all genotypes identified were high-risk).

In conclusion, our study showed that α-HPV positivity in NMSC and MSC was 32.50%. In our lot of patients, 40% of SCC were HPV positive, 35% of BCC were HPV positive and 20% of MM were HPV positive. Comparative analysis of α-HPV positive skin tumors (32.50%) and α-HPV positive control group (15%) revealed a higher positivity of α-HPV in the tumor group with a ratio of 2.16.

In our study, the samples for HPV DNA detection and typing were randomly selected. The number of investigated cases was limited; however, it is similar to the number of samples analyzed in other studies focusing on α-HPV infection in cutaneous cancer (20).

Future studies about the incidence/prevalence of HPV infections in skin cancer are desirable in our country, since knowledge about these aspects is limited.

In order to assess the role played by HPV (determinant or contributory), we recommend that further studies be conducted in order to correlate HPV positivity with other known risk factors of skin cancers.

Conflicts of interest

There are no conflicts of interest for any of the authors.

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Abbreviations

HPV – human papillomaviruses
MSC – melanoma skin cancers
NMSC – non-melanoma skin cancers
DNA – deoxyribonucleic acid
PCR – polymerase chain reaction
BCC – basal cell carcinoma
SCC – squamous cell carcinoma
MM – malignant melanoma
GP – general primer
dNTPs – deoxynucleotide triphosphates
RNase - ribonuclease

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