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ORIGINAL ARTICLE

Investigation of three oncogenic epitheliotropic viruses shows human papillomavirus in association with non-melanoma skin cancer

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Abstract

Non-melanoma skin cancers (NMSC) share similar risk factors with other virus-related cancers, despite the lack of proved causal association between viral infection and NMSC development. We investigated the presence of Merkel cell polyomavirus (MCPyV), Epstein-Barr virus (EBV), and human papillomavirus (HPV) DNA in 83 NMSC fresh-frozen and 16 non-cancerous skin biopsies and evaluated viral infection according to demographical data, histopathological diagnosis, and ultraviolet exposure. Our results showed that 75% of NMSC biopsies were positive for at least one out of three viruses, whereas only 38% of non-cancerous skin biopsies were positive ($p = 0.02$). Notably, HPV detection was frequent in NMSC (43%) and nearly absent (one sample, 6.7%) in non-cancerous biopsies ($p = 0.007$). MCPyV was associated with sites of higher exposure to ultraviolet radiation ($p = 0.010$), while EBV was associated with a compromised immune system ($p = 0.032$). Our study showed that HPV was strongly associated with NMSC while EBV and MCPyV with other risk factors. Though further studies are required to elucidate the role of viral infection in NMSC development and management, this study supports the possible role of oncogenic viruses in skin cancers, especially HPV.

Keywords Skin cancer · Oncogenic viruses · Viral detection · Biopsy

Introduction

Non-melanoma skin cancers (NMSC) are the most prevalent cancers worldwide, especially among Caucasian populations. In Brazil, a highly mixed-race country, the estimated incidence of NMSC is about 170/100,000 cases [1]. Although NMSC comprehends a large variety of cutaneous neoplasia, the vast majority of them are classified as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [2, 3]. In addition to SCC and BCC, NMSC include Merkel cell carcinoma (MCC), an aggressive neuroendocrine cutaneous neoplasia etiologically linked to the recently discovered Merkel cell polyomavirus (MCPyV) [4].

Despite the diversity of histopathological subtypes, skin cancers are usually caused by cellular transformation due to the accumulation of ultraviolet-related DNA mutations [5–8]. Other risk factors for NMSC include immunological impairment and possibly persistent infections of oncogenic viruses [9]. The involvement of the latter in skin cancer is challenging to prove since viruses with oncogenic potential are usually found both in healthy and neoplastic samples [10]. For instance, studies on the association between NMSC and cutaneous human papillomavirus (HPV)—one of the most studied virus in NMSC—have shown controversial results [11, 12]. Nevertheless, the discovery of Merkel cell polyomavirus in

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MCC has renewed scientific interest on viral causality in neoplasia. Additionally, most studies on viral infection in NMSC focused on only one virus at a time, such as either HPV or MCPyV, and most of them have not investigated other oncogenic viruses with epithelial tropism, such as the Epstein-Barr virus (EBV).

Therefore, our goal was to investigate the occurrence of three oncogenic epitheliotropic viruses, MCPyV, EBV and HPV, in both non-cancerous skin and NMSC biopsies, and to analyze associations between viral infection and known risk factors for NMSC.

Material and methods

Samples

A transversal study was conducted using 83 fresh-frozen NMSC biopsies from patients attended by the Dermatological Service of the Antônio Pedro University Hospital (HUAP/UFF) between January 2014 and May 2016, with clinical indication of cutaneous biopsy for histopathological diagnosis. For the sake of comparison, 16 non-cancerous skin tissue biopsies were also collected during this period at the same hospital. One biopsy per patient was used in this study. All individuals agreed on participating by signing an informed consent.

Data on gender, age, tumor location, immunological status, and ethnicity were collected in the medical examiner interview. Ethnicity was defined by a dermatologist, according to the phototype of the patient. For such, the skin reaction to sun exposure and patient's original hair and eye colors were considered to classify their ethnicity into only two groups, "white" or "non-white."

DNA extraction from biopsies and molecular diagnosis

Biopsies were fragmented, digested by proteinase K (Promega®—Madison, USA), and DNA was extracted by RTP® DNA/RNA Kit (Molecular Stratec Biomedical—Berlin, Germany) according to manufacturer instructions. Viral DNA was detected by nested-PCR for the LT3 region of MCPyV [13]; EBNA2 region for EBV [14]; and generic MY9/M11 for HPV [15]. PCR products were visualized on a 2% agarose gel electrophoresis stained with ethidium bromide.

Statistical analysis

Statistical analysis was performed using SPSS statistics 20 software (SPSS, Inc., Chicago, IL). The chi-squared test and the Mann-Whitney test were used to examine associations between viral detection, demographical data, immune status, and lesion location.

Results

In the NMSC group, basal cell carcinoma (BCC) was the most frequently found histopathological type (74%), followed by squamous cell carcinoma (SCC) (6%), actinic keratosis (3.6%), Bowen's disease (1.2%), and others. Tumor biopsies predominated on frequently and moderately sun-exposed areas (91.6%—head, neck, and limbs). Of the 16 non-cancerous biopsies, 10 derived from skin grafts used to close the surgical wound, two were obtained in plastic surgery, two were from benign cysts, one from actinic elastosis (a benign outcome to photoaging), and one from an extended resection margin of a previously operated melanoma.The majority of the 99 skin tissue samples from this study were classified as originated from white (86.9), male (56.6%), and elderly (82.8%) (over 60 years old) patients.

Regarding the four immunocompromised NMSC patients, two have kidney transplants, one has myelodysplasia, and one has HIV infection. None of the non-cancerous patients were immunosuppressed. In order to evaluate comparability between NMSC and non-cancerous groups, statistical analyses determined that ethnicity, gender, age, and solar exposure did

Table 1 Comparison between non-melanoma skin cancer ($N = 83$) and non-cancerous ($N = 16$) groups with demographic, solar exposure, immune status, and viral detection

Variable		Non-melanoma skin cancer (%)		
		Present	Absent	*P*value
Gender	Male	57.8	50.0	0.563
	Female	42.2	50.0	
Ethnicity	White	81.2	88.0	0.486
	Non-white	18.8	12.0	
Age	Average (±SD)	72 (12.2)	67 (15.7)	0.411
Solar exposure	Low	8.4	12.5	0.465
	Intermediate	16.9	6.2	
	High	74.7	81.2	
Immunocompromised	Absent	94.9	100.0	0.219
	Present	5.1	0	
MCPyV	Absent	60.2	68.8	0.522
	Present	39.8	31.2	
EBV	Absent	69.9	87.5	0.122
	Present	30.1	12.5	
HPV	Absent	56.6	93.3	*0.007*
	Present	43.4	6.7	
Multiple detections	Absent	73.3	100.0	*0.003*
	Present	26.7	0	

MCPyV Merkel cell polyomavirus,*EBV* Epstein-Barr virus,*HPV*human papillomavirus;$p \leq 0.05$ results are in italics

Table 2 Viral detection according to demographic, solar exposure, and immune status of all patients ($N = 99$) and NMSC only (N = 83) [in brackets]

Variable		Viral frequency (%)					
		MCPyV	*p*value	EBV	*p*value	HPV	*p*value
Gender	Male	30.4 [31.2]	0.061 [0.064]	32.1 [33.3]	0.214 [0.455]	35.3 [40.9]	0.645 [0.604]
	Female	48.8 [51.4]		20.9 [25.7]		40.0 [46.9]	
Ethnicity	White	39.5 [41.1]	0.540 [0.495]	30.2 [32.9]	0.059 [0.106]	35.4 [40.3]	0.338 [0.135]
	Non-white	30.8 [30.0]		7.7 [10.0]		50.0 [66.7]	
Age	Average (±SD)	71 (11.7) [72(10.1)]	*0.052*[*0.035*]	68.1(12.1) [70.4(12.2)]	0.272 [0.357]	69.6 (13.6) [67.8(12.2)]	0.794 [0.960]
Solar exposure	Low	0 [0]	*0.010* [*0.023*]	11.1 [14.3]	0.322 [0.387]	33.3 [42.9]	0.573 [0.977]
	Moderate	40.0 [42.9]		20.0 [21.4]		50.0 [46.2]	
	High	42.7 [43.5]		30.7 [33.9]		35.3 [42.9]	
Immunocompromised	Absent	38.5 [40.0]	0.576 [0.538]	23.1 [25.3]	*0.032* [*0.044*]	36.1 [42.6]	0.581 [0.744]
	Present	25.0 [25.0]		75.0 [75.0]		50.0 [50.0]	

MCPyV Merkel cell polyomavirus,*EBV* Epstein-Barr virus,*HPV*human papillomavirus; $p \leq 0.05$ results are in italics

not vary significantly between them (Table 1). Among the viruses investigated, only HPV was significantly found in NMSC (43%; $p = 0.007$), being detected in only one (6.7%) non-cancerous biopsy. Multiple viral detections occurred less frequently than single detections in NMSC biopsies (26.7% vs 73.3%) but were significantly higher than non-cancerous skin biopsies ($p = 0.003$) in which no multiple detections were found.

Viral frequencies according to demographical data, immune status, and solar exposure are shown in Table 2. Overall, the presence of EBV DNA was associated with immunocompromised patients ($p = 0.032$), though a trend toward an association with fair skin was observed as well ($p = 0.059$). MCPyV detection was statistically higher in UV-exposed biopsies ($p = 0.010$). Also, MCPyV has shown to have a statistical tendency toward the elderly ($p = 0.052$) and male populations ($p = 0.061$). When the same associations were evaluated inside the NMSC group alone, similar results were found, except for the significant detection of MCPyV in skin biopsies of elderly patients ($p = 0.035$).

Discussion

In this study, we evaluated the molecular detection of three oncogenic viruses MCPyV, EBV, and HPV in NMSC and non-cancerous samples and found a high HPV detection rate in NMSC ($p = 0.007$). This finding is particularly interesting for it incites the hypothesis of a viral role in NMSC oncogenesis or tumor maintenance. Moreover, the near absence of HPV in non-cancerous skin is surprising, considering previous studies have detected HPV in both NMSC patients and healthy controls [16, 17]. Such disparity in prevalence must be further investigated to elucidate the possible effect of HPV in NMSC oncogenesis and development. For instance, a meta-analysis with 45 studies presented a mean β-HPV prevalence of 40% in BCC (the most frequent NMSC), which corroborates our findings, and 44.4% of positivity in controls. Similar prevalences were also observed in both NMSC and controls regarding α and γ-HPV but not for *epidermodysplasia verruciformis* associated HPV (EV-HPVs). The EV-HPV types were more prevalent in NMSC than controls [18]. On top of having skin tropism, the β-genus HPV, especially the types 5, 8, 17, 20, 24, and 38, are found to increase the risk of SCC development up to 45% [19]. Unfortunately, it was not possible to perform genotyping due to low DNA quantity and sample constraints. However, it is important to notice that β-genus HPV cannot be classified according to their oncogenicity due to a paucity of information.

Future studies addressing whether the β-HPV detected in these cases are actually localized within the malignant cells or whether they are transcriptionally active would greatly strengthen the evidence for a carcinogenic role of these

pathogens. On the other hand, they may act as a cofactor that enhances the carcinogenic potential of UV damage, as shown by Wallace et al., who also demonstrated that β-HPV E6 expression can enhance the carcinogenic potential of UV exposure by promoting p300 degradation and tolerating genetic instability [20, 21].

Our results might have interesting future implications in NMSC prevention and treatment, once they are further confirmed. Since HPV already has a vaccine approved and effective against cervical cancer, studies should evaluate whether this vaccine could decrease NMSC development in risk populations. Furthermore, intratumoral treatment with a 9-valent HPV vaccine could be an alternative to surgery, the standard treatment. A recent study showed a total regression of cutaneous SCC lesions in one elderly patient after intratumoral vaccination [22]. Such alternative to surgery, though innovative and still lacking further proved effectiveness in large clinical trials, might represent an important improvement to NMSC treatment and endorses HPV detection and genotyping relevance in these patients.

Recent studies suggest that viral infection in non-melanoma skin cancers may have a potential co-carcinogenic effect, along with the ultraviolet radiation [6, 23–25]. In fact, we herein report the first association of MCPyV detection with NMSC from sun-exposed areas. This result corroborates the co-carcinogen hypothesis [4, 23, 26, 27], though it should be confirmed by additional studies. On the other hand, the higher frequency of MCPyV amongthe elderly may be a consequence of immune senescence, where higher levels of viral shedding from skin tissue could be expected. Furthermore, the statistical association found among EBV detection in skin samples and immunocompromised patients is unprecedented, although not totally unexpected due to the opportunistic behavior of herpesviruses in general, but requires further studies to be fully elucidated.

In spite of the fact that multiple viral detection rates were lower than single infections, coinfections were also found strongly associated with NMSC. This could probably be explained by the high HPV frequency in the skin rather than a real synergetic effect during multiple infections. Nevertheless, in vitrostudies showing how these viruses would behave in simultaneous infections could give insights into coinfection relevance in vivo.

The reduced sample size, especially in the non-cancerous group, may have limited further analysis, although such discrepancy was observed in other studies where smaller control groups were employed [28, 29]. Also, the low DNA concentration prevented HPV genotyping. The lack of HPV genotyping confers a considerable limitation to this study hindering further analysis on NMSC risks related to specific HPV genus. Another important limitation is the chosen method for HPV detection, which may not be optimal for cutaneous HPV detection and could have potentially led to an underestimation of β-HPV infection. However, MY09/11 primers are able to identify cutaneous HPV infection, though in a limited extent [30, 31]. Ideally, the samples would have been further tested for cutaneous HPV through a specific set of primers [31], but, as stated previously, the small amount of sample and DNA available for all viral detection rendered further specific analysis unfeasible. Nevertheless, there is compelling evidence of HPV infection in NMSC but not in non-cancerous skin in this study, which argues that even if β-HPV infection might be underestimated, there still is an association between HPV infection and NMSC.

Despite such limitations, this study presents some novel associations between viral infections in the skin tissue, pointing to a possible association between HPV and skin cancer. Such information albeit partial can provide a foundation to which future studies can build upon. Although frequent viral detection in NMSC is not sufficient evidence to establish causality, it surely instigates further and larger researches on how viral infection could influence NMSC development and maintenance.

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Compliance with ethical standards

Conflict of interest The authors declare that they haveno conflict of interest.

Ethical approval The study was approved by the University Hospital Ethical Committee (protocol 608.880/2014). Informed consent was obtained from all individual participants included in the study.

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