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Detection of Merkel cell polyomavirus in tumor cells and peritumoral lymphocytes of non-melanoma skin cancer by Immunohistochemistry

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Abstract

Merkel cell polyomavirus (MCPyV) is an oncogenic virus etiologically related to Merkel cell carcinoma and has been found in association with non-melanoma skin cancer (NMSC), with controversial results. Little is known about the role or influence of MCPyV in the development of these tumors and its prevalence in various populations.

To assess the prevalence of MCPyV`s Large T Antigen and its DNA in cases of NMSC, nested-PCR and immunohistochemistry (IHC) were performed on 103 samples of NMSC from 91 patients and these results were correlated with epidemiological and demographical data. A total of 44 (42,7%) tumors were positive for the virus via PCR, 34 (77,3%) in the BCC group, 6 (13,6%) in the cSCC, 3 (6,8%) for Bowen's disease and 1 (2,3%) for keratoacanthoma. The IHC results shows a positivity of 15 (14,6%), 7 cases of positivity in the tumor, 8 in the peri-tumoral infiltrate and 3 in both, and with 11 (73,0%) of positivity in the BCC group, 3 (20,0%) in the cSCC, 0 (0,0%) for Bowen's disease and 1 (7,0%) for keratoacanthoma.

Despite the frequent detection of MCPyV DNA and large T antigen in NMSC, its possible role in the development of NMSC is unlikely.

Keywords: Cancer Viruses; Viral Cancer; Merkel cell polyomavirus (MCPyV); Immunohistochemistry (IHC)

1. Introduction

Merkel cell carcinoma (MCC) is a rare, neuroendocrine, aggressive cutaneous tumor, with well-established virus involvement in about 80% of the cases, named merkel cell polyomavirus (MCPyV), a human polyomaviruses (HPyVs). In virus-positive tumors, MCPyV is integrated into the human genome, and the viral oncoproteins large T-antigens are implicated on multiple pathways of cell growth, including inhibition of tumor-suppressor retinoblastoma protein, stabilization of oncoprotein c-Myc and evasion of innate immunity [1]. The clonal integration of the virus with the tumor cells appear to be a driving force in MCC development, as the MCPyV genome is found as a nonintegrated episome in MCPyV carriers without MCC [2].

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Since 2008, after the virus description by Feng and colleagues [1], its genome has been founded in several nonmelanoma skin cancers (NMSC) using PCR technique, mainly basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC), with controversial results [3].

At the present moment, there is no gold standard assay for the detection of the merkel cell polyomavirus (MCPyV), even with a multimodal method, the detection of MCPyV in tumor samples remains suboptimal with both immunohistochemistry (IHC) and quantitative PCR (qPCR) approaches [4].

Serologic studies suggest that a substantial fraction of adult human beings, with a rate of 80% among persons older than 50 years, may be asymptomatically infected with MCPvV [5]. The infection occurs early in life, with 60% to 80% of children testing positive for antibodies against MCPyV by 4 to 5 years of age [2]. The viral DNA sequences also have been traced in various nonmalignant tissues such as skin, aerodigestive tract and genitourinary system [6,7]. The widespread presence of the virus could lead to the interpretation of a casual, incidental infection of BCC and cSCC tumor cells.

2. Material and methods

This study met the local ethical guidelines and was approved by Ethical Research Committee of the Fluminense Federal University's Hospital. Participants provided signed informed consent.

Fresh-frozen resections collected between January 2014 and November 2019 were used for this study. Data on patient sex, age and ethnicity and tumor location were collected through patient interview. The surgical procedure was performed according to equivalent international standards for different NMSCs, providing tissue material for histopathological diagnosis and molecular procedures. The samples were immediately frozen in RNAlater Stabilization Solution (Thermo Fisher Scien- tific Inc., Waltham, MA, USA) at -80 °C until further molecular analysis.

All 103 cases of non-melanoma skin cancer were collected from the University Hospital Antônio Pedro that belongs to the Federal Fluminense University. These cases were available in paraffin blocks and identified by searching the Pathology department databases from 2014 to 2019 and were retrieved and reviewed by an experienced pathologist (MCR and LP) to confirm the type and subtype diagnosis.

2.1. Immunohistochemistry and Histopathological diagnosis

Skin fragments were sent for routine histological processing at the Anatomic Pathology Division, Fluminense Federal University. Immunohistochemical staining for MCPyV (Clone cm2b4; 1/100 dilution; Santa Cruz, USA) was performed according to standard laboratory protocols. MCPyV immunohistochemistry was considered positive if any nuclear staining was identified, with positive cases graded in a semiquantitative fashion on a two point scale: 1, weak staining; 2, strong staining. A score of 1 or more was considered positive for the purposes of statistical evaluation, the same with the distribution of the staining, if focal or diffuse. Positive controls were run in parallel.

2.2. DNA extraction and detection of Merkel cell polyomavirus DNA by PCR

Merkel cell polyomavirus DNA extraction from fresh-frozen tissue were individually macerated with the use of a sterile disposable scalpel and digested with proteinase K (Promega", Madison, WI, USA), DNA was subsequently extracted (RTP" DNA/RNA Virus Mini Kit; Stratec Molecular Biomedical, Berlin, Germany) in accordance with the manufacturer's instructions. Nested PCR (nPCR) for LT3 of MCPyV was performed as described previously [8]. PCR product was confirmed by DNA sequencing upon establishing the protocol.

2.3. Statistical Analysis

The differences between proportions and Associations between categorical variables were treated by the chi-square test. Differences between groups of numerical variables were investigated by Student's t test after the observation of normality by the Kolmogorov-Smirnov test.

3. Results and discussion

There were 91 patients with non-melanoma skin cancer for whom tissue of 103 tumors was available. Average age was 69.3 (+/-13,16) years and most of the patients, 81 (88.3%), were white. 51 (49.5%) patients were female and 52 (50.5%) were male. 76 tumors (73.8%) were located on the head and neck, 11 (10.7%) on the limbs, and 16 (15.5%)

on the trunk. A total of 92 (89.3%) were considered to be located in sun-exposed sites and 11 (10.7%) in non-sun-exposed sites.

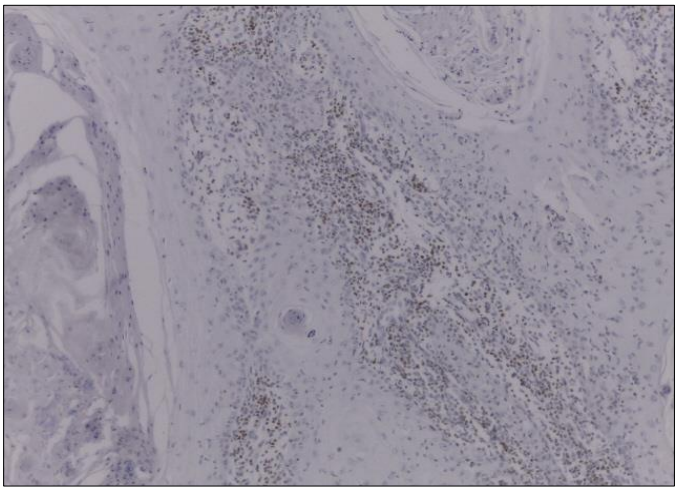

Figure 1 Immunohistochemistry for CM2B4 antibody with nuclear positivity in peri-tumoral lymphocytic infiltrate in a cutaneous squamous cell carcinoma x100

Table 1 Comparison between the presence (n=44) and absence (n=59) of MCPyV DNA by PCR detection with demographics data, tumor location and type, solar exposure and immunohistochemistry results.

Variable		**MCPyV frequency by PCR (%)**		
		Present	**Absent**	***P*Value**
Gender	Male	(19) 43.2	(33) 55.9	0.235
	Female	(25) 56.8	(26) 44.1	
Ethnicity	White	(41) 93.2	(50) 84.7	0.435
	Black	(1) 2.3	(1) 1.7	
	Pardo(mixed ethnicity)	(2) 4.5	8 (13.6)	
Age	Average (±sd)	69.0 (13.95)	69.5 (± 12.65)	0.868
Location	Head and neck	(31) 70.5	(45) 76.3	0.490
	Trunk	(4) 9.1	(7) 11.9	
	Limbs	(9) 20.5	(7) 11.9	
Tumor type	BCC	(34) 77.3	(51) 86.4	0.573
	sSCC	(6) 13.6	(6) 10.2	
	Bowen's disease	(3) 6.8	(1) 1.7	
	keratoacanthoma	(1) 2.3	(1) 1.7	
Solar Exposure	Yes	(40) 90.9	(52) 88.1	0.755
	No	(4) 9.1	(7) 11.9	
IHQ MCPyV	Negative in both	(40) 90.9	(45) 76.2	0,145
	Positive in the tumor	(1) 2.3	(6) 10.2	
	Positive in the peri-tumoral lymphocytic infiltrate	(2) 4.5	(6) 10.2	
	Positive in both	(1) 2.3	(2) 3.4	

A total of 44 (42.7%) tumors were positive for the virus presence by the PCR, with 34 (77.3%) of positivity in the BCC group, 6 (13.6%) in the cSCC, 3 (6.8%) for Bowen's disease and 1 (2.3%) for keratoacanthoma (Table 1). The IHC results shows a total positivity of 18 (17.5%), with 7 cases of positivity in the tumor, 8 in the infiltrate (Figure 1) and 3 in both (Figure 2). There was 13 (72.2%) of positivity in the BCC group, 4 (22.2%) in the cSCC, 0 (0.0%) for Bowen's disease and 1 (5.6%) for keratoacanthoma (Table 2).

The correlation between PCR and IHC approaches was 3.0% for positive samples and 43.7% for negative. Histological subtype were also taken into account, for the BCC, the superficial and nodular (low risk group) had 21 (61.7%) of positivity for the virus by PCR and 11 (64.7%) by IHC, as the sclerosing, the infiltrating, the micronodular and the metatypical subtypes (high risk group) had 13 (38.2%) of positivity for the virus by PCR and 2 (25.0%) by IHC, for the cSCC, well-differentiated (low risk group) had 4 (66.7%) of positivity for the virus by PCR and 2 (50.0%) by IHC, as the moderately-differentiated and poorly-differentiated (high risk group) had 2 (33.3%) of positivity for the virus by PCR and 2 (50.0%) by IHC.

Table 2 Comparison between the absence (n=85) and presence (n=18) of MCPyV`s Large T Antigen by Immunohistochemistry with patient demographics data, tumor location and type, solar exposure and PCR results.

Variable		**IHC MCPyV**				
		Negative	**Positive in the Tumor**	**Positive in the peri-tumoral lymphocytes**	**Positive in both**	***P* value**
Gender	Male	(40) 81.6	(2) 3.8	(7) 13.5	(3) 5.8	0.017
	Female	(45) 88.2	(5) 9.8	(1) 2.0	(0) 0.0	
Ethnicity	White	(76) 83.5	(7) 7.7	(5) 5.5	(3) 3.3	0.113
	Black	(1) 50.0	(0) 0.0	(1) 50.0	0 (0.0)	
	Pardo(mixed Ethnicity)	(8) 80.0	(0) 0.0	(2) 20.0	0 (0.0)	
Age	Average (±sd)	69.7 (±13.29)	65.7 (± 11.76)	66,5 (± 14,71)	75,5 (± 9,19)	0.728
Location	Head and neck	(64) 84.2	(6) 7.9	(4) 5.3	(2) 2.6	0.422
	Trunk	(8) 72.7	(0) 0.0	(2) 18.2	(1) 9.1	
	Limbs	(13) 81.3	(1) 6.3	(2) 12.5	(0) 0.0	
Tumor type	BCC	(72) 84.7	(6) 7.1	(2) 5.9	(2) 2.4	0.388
	cSCC	(8) 66.7	(1) 8.3	(2) 16.7	(1) 8.3	
	Bowen's disease	(4) 100.0	(0) 0.0	(0) 0.0	0 (0.0)	
	Keratoacanthoma	(1) 50.0	(0) 0.0	(1) 50.0	0 (0.0)	
Solar exposure	Yes	(77) 83.7	(7) 7.6	(6) 6.5	(2) 2.2	0.158
	No	(8) 72.7	(0) 0.0	(2) 18.2	(1) 9.1	
PCR	Negative	(45) 76.3	(6) 10.2	(6) 10.2	(2) 3.4	0.285
	Positive	(40) 90.9	(1) 2.3	(2) 4.5	(1) 2.3	

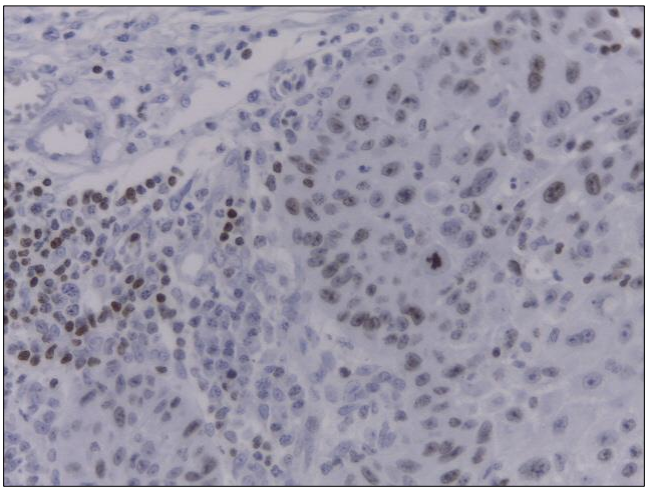

Figure 2 Immunohistochemistry for CM2B4 antibody with nuclear positivity in both tumor cells and peri-tumoral lymphocytic infiltrate in a cutaneous squamous cell carcinoma x 400

Prior studies have used PCR approaches in addition to IHC to detect MCPyV in MCC samples, with few authors inquiring its presence in other skin cancers. These studies detected virus in more samples by PCR than by IHC, suggesting that PCR detection is more sensitive [9,10,11,12].

In contrast, other authors detected the virus in more MCC tumors using IHC than with their PCR assay. This suggests that some PCR assays, whether due to the detection of different DNA regions, utilization of different primer sets, or, in the case of quantitative PCR utilizing different thresholds to consider a test positive, yield different sensitivities compared to IHC. Therefore, it is possible that the most sensitive and specific PCR assay for MCPyV detection has yet to be compared with IHC assays [4].

Reisinger and colleagues studied 12 patients with MCC that has developed a subsequent NMSC, being 9 BCC and 3 cSCC. Although 11 of the 12 MCC tumors from patients with secondary tumors were positive for MCPyV T antigen, none of the secondary BCC or cSCC showed evidence for CM2B4 reactivity [13]. In consonance, Mertz and colleagues described a total of 163 patients with NMSC and skin controls, showing a high rate of virus detection by PCR using two different primer pairs, ranging from 39.4% to 47.1% in BCC from immunocompetent and immunosuppressed patients, and 28.3% to 26.7% in cSCC from immunocompetent and immunosuppressed patients, respectively. However, the detection rate in the pooled group of NMSC, only three tumors (1.8%) were positive by immunohistochemistry [8].

An IHC based survey performed by Toptan and colleagues, using an antibody cocktail for HPyVs that includes MCPyV, screened hundreds of tumors from different sites, including skin malignancies such as BCC and cSCC. No evidence of the presence of MCPyV in these tumors was found, and rare cells showing HPyV infection in some of lung carcinomas cases were interpreted likely to represent incidental or passenger infection [14].

It has been suggested in the Australian population that the MCPyV virus may be a less important cause of MCC neoplasia due to the highly sun-exposed and predominantly fair-skinned characteristics of this population [15]. This thought also applied to the finding that MCC with mixed squamous differentiation is often negative for MCPyV, since it occurs in more heavily sun-exposed areas of the body leading to the hypothesis that the sun exposure may drive MCC through an alternative pathway to the virus-driven oncogenesis [9,16].

The rate of MCPyV positivity seen in tumors from sun-damaged and nonsun-damaged skin has been studied as well by Dabner and colleagues, using as histological parameters the dermal elastosis and the presence of epidermal dysplasia as markers of actinic damage. The results show a less prevalence of the virus in tumors resulting from UV exposure sites [17]. Nevertheless, recent studies report the opposite situation, with a higher viral presence in NMSC located in sun-exposed locations, suggesting that viral infection along with chronic ultraviolet radiation in NMSC may have a potential co-carcinogenic effect [18]. The results of the present study did not show a relevant difference between the presence by PCR of the MCPyV in sun-exposed and non-sun-exposed sites, not being able to reinforce the hypothesis of different pathways of carcinogenesis in these tumors. It is known that the monoclonal anti-MCPyV antibody CM2B4, developed by Shuda and colleagues [19] that recognizes the virus large T antigen, is highly sensitive and specific for the presence of a high-viral copy number in cells, nevertheless, it is commonly negative in tissue with a low-viral copy number [9,16],

which could explain the samples with positive PCR and negative IHC. The viral positivity in the peri-tumoral lymphocytic infiltrate of these tumors is still not fully understood and rarely described [20]. Immunostaining was observed in some lymphocytes and mast cells by Leroux-Kozal et al, with no further information about the correlation with the IHC positivity [11]. Low-level staining of tonsillar tissue by CM2B4 was occasionally described by Moshiri and colleagues, and it was considered non-specific, not affecting the interpretation of tumor tissue staining by the authors [20].

The absence of additional antibodies recognizing the MCPyV, despite the high specificity of the CM2B4 antibody, also limits the interpretation of the immunostaining in these cells since and it could represent just a non-specific signaling.

Nevertheless, considering high specificity and sensitivity of the CM2B4 antibody, the IHC results may reinforce the hypothesis of a viral presence unrelated to the genesis of these tumors, due to the virus ubiquity. Although these results demonstrate a very low correlation between PCR and IHC results, the positivity in the lymphocytic infiltrate would also explain cases with positive PCR in which there would be no probable causal relationship between the virus and the carcinoma. The PCR positive samples with negative IHC may be explained by the low sensitive and specificity for tissues with a low-viral copy number, which may be the case in the vast majority of these samples. However, the positive IHC samples with negative PCR results may be explained by a low specificity of this method in these NMSC tissues.

Previous studies in MCC tumors usually show strong correlation between the detection and amount of MCPyV DNA by real-time PCR and the LTA expression by IHC, reinforcing that these 2 methods could be used as complementary to each other. However, the low correlation between the results of the different techniques in this study encourages the authors to consider some hypothesis: that the IHC has low specificity and is not an ideal test for the detection of MCPyV in non-merkel cell carcinoma tumors; that these techniques, although complementary to each other, could not yet be considered a gold standard clinical test to detect MCPyV.

4. Conclusion

Due to the low prevalence of the virus in these NMSC samples and poor correlation between MCPyV presence by PCR and IHC techniques, the authors considers that is still no gold standard assay for the detection of the merkel cell polyomavirus, even with a multimodal method, and that its possible role in the development of NMSC is unlikely. However, additional analyses including the viral DNA sequencing, genome integration of the MCPyV to these tumors, as well as the use of additional antibodies recognizing the MCPyV small or large antigens by IHC are necessary.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors Thiago Rubim Batista Bellott Nascimento, Flávio Barbosa Luz, Anna Karoline Fausto da Silva, Rafael Brandão Varella, Maria Angelica Arpon Marandino Guimarães, Marianna Tavares Venceslau Gonçalves, Mayra Carrijo Rochael and Luciana Pantaleão, have no conflicts of interest to declare.

Statement of ethical approval

This study met the local ethical guidelines and was approved by Ethical Research Committee of the Fluminense Federal University's Hospital.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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