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Cesare Campello, Manola Comar, Pierlanfranco d'Agaro, Anna Minicozzi,  
Luca Rodella, Albino Poli

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## A MOLECULAR CASE-CONTROL STUDY ON THE MERKEL CELL POLYOMAVIRUS IN COLON CANCER

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Complete List of Authors:	<p>Campello, Cesare; University of Trieste, Department of Reproductive, Developmental and Public Health Sciences, and IRCCS Burlo Garofolo</p> <p>Comar, Manola; University of Trieste, Department of Reproductive, Developmental and Public Health Sciences, and IRCCS Burlo Garofolo</p> <p>D'Agaro, Pierlanfranco; University of Trieste, Department of Reproductive, Developmental and Public Health Sciences, and IRCCS Burlo Garofolo</p> <p>Minicozzi, Anna; University of Verona, Department of Anaesthesiology and Surgery</p> <p>Rodella, Luca; University of Verona, General Surgery Unit, Endoscopy Service</p> <p>Poli, Albino; University of Verona, Department of Medicine and Public Health</p>
Keywords:	Merkel cell polyomavirus, colon cancer, molecular case-control study

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Table I. Main pathological features of the cases and controls, and positive results for Merkel cell polyomavirus

	n.	%	** MCPyV positive	
			n. (%)	(95% CI)
<b>CONTROLS</b>				
No lesion	52	65.0	4 (7.7)	2.09 – 19.70
Adenoma	15	18.8	2 (13.3)	1.61 – 48.17
Hyperplastic polyp	9	11.2	1 (11.1)	0.28 – 61.91
Other conditions	4	5.0	0	--
<b>Total</b>	<b>80</b>	<b>--</b>	<b>7*(8.8)</b>	<b>3.52–18.03</b>
<b>CASES</b>				
Adenocarcinoma	57	89.0	4 (7.0)	1.92 – 16.1
Mucinous	6	9.4	0	--
Adenocarcinoma	1	1.1	0	--
Neuroendocrine cancer	1	1.1	0	--
<b>Total</b>	<b>64</b>	<b>--</b>	<b>4* (6.3)</b>	<b>1.71 – 16.01</b>

\*Fisher exact test: P=0.755

\*\* MCPyV = Merkel cell polyomavirus

Table II. Nucleotide variation in the viral protein 1 (VP1) gene of two representative Merkel cell polyomavirus sequences (in bold) in comparison with a number of GenBank registered sequences.

Strain	GenBank Accession N.	VP1 gene - Nucleotide position				Country
		3825	3875	3909	4023	
MCC350	EU375803	T	C	C	T	USA
MCC339	EU375804	<b>C</b>	<b>G</b>	<b>T</b>	<b>A</b>	USA
MKT-23	FM864208	<b>C</b>	<b>G</b>	C	T	France
MKT-31	FM864210	<b>C</b>	C	C	T	France
VP1-Mpt2	AM992903	<b>C</b>	<b>G</b>	C	<b>A</b>	France
<b>MCV ITA 61</b>	<b>HQ262570</b>	<b>C</b>	<b>C</b>	<b>C</b>	<b>T</b>	<b>Italy</b>
<b>MCV ITA 71</b>	<b>HQ262569</b>	<b>C</b>	<b>G</b>	<b>C</b>	<b>T</b>	<b>Italy</b>

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4 1 A MOLECULAR CASE–CONTROL STUDY ON THE MERKEL  
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11 4 Cesare Campello<sup>1</sup>, Manola Comar<sup>1</sup>, Pierlanfranco D’Agaro<sup>1</sup>, Anna  
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13 5 Minicozzi<sup>2</sup>, Luca Rodella<sup>3</sup>, and Albino Poli<sup>4</sup>  
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20 7 **Affiliations.** <sup>1</sup>Department of Reproductive, Developmental and Public  
21  
22 8 Health Sciences, University of Trieste, and IRCCS Burlo Garofolo,  
23  
24 9 Trieste, Italy; <sup>2</sup>Department of Anaesthesiology and Surgery, University of  
25  
26 10 Verona, Italy; <sup>3</sup>General Surgery Unit, Endoscopy Service, Teaching  
27  
28 11 Hospital of Verona; <sup>4</sup>Department of Medicine and Public Health,  
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30 12 University of Verona.  
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36 13 **Running title.** Merkel polyomavirus and colon cancer  
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39 14 **Key words.** Merkel cell polyomavirus; colon cancer; molecular case–  
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41 15 control study  
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46 17 **Correspondence to:** C. Campello, Department of Reproductive,  
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48 18 Developmental and Public Health Sciences, University of Trieste, Via  
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50 19 dell’Istria 65/1, 34137 Trieste, Italy. Phone +39-040-773433, Fax +39-  
51  
52 20 040-7600324; e-mail: [campello@burlo.trieste.it](mailto:campello@burlo.trieste.it).  
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60 24 **Word count:** 1945

## 1 Abstract

2 To explore the putative role of the Merkel cell polyomavirus in human  
3 colon cancer, a prospective molecular case–control study was undertaken  
4 in patients and their relatives enrolled during a screening programme.

5 Fresh tissue samples from 64 cases of colon cancer (mean age  $69.9 \pm 11.0$   
6 years; 40 males) and fresh biopsies from 80 relatives (mean age  $53.7 \pm 8.6$   
7 years; 43 male; 55 son/daughter, 23 brother/sister, 2 parents) were  
8 analysed by PCR and sequencing. Precancerous lesions, namely  
9 adenomas and polyps, were detected in 15 (18.8%) and 9 (11.2%) of the  
10 controls, respectively. In addition, 144 blood samples were examined.

11 Merkel cell polyomavirus DNA was detected in 6.3% of cases and 8.8%  
12 of controls. This difference was not statistically significant in the logistic  
13 regression analysis, after adjustment for age. Whereas blood samples  
14 from both cases and controls tested negative, the DNA Merkel cell  
15 polyomavirus was identified in 12.5% of adenoma/polyp tissues. No  
16 statistically significant difference was found when prevalence rates of  
17 Merkel cell polyomavirus in normal, precancerous and cancer tissues  
18 were compared.

19 Sequence analysis of the viral LT3 and VP1 regions showed high  
20 homology (>99%) with those of strains circulating worldwide, especially  
21 with genotypes detected in France.

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4 1 The findings of this survey are consistent with the hypothesis that the  
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6 2 Merkel cell polyomavirus, in addition to other human polyomaviruses,  
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9 3 can be recovered frequently from the gastrointestinal tract, because it is  
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12 4 transmitted throughout the faecal–oral route. Moreover, the study does  
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15 5 not indicate a role for Merkel cell polyomavirus in the genesis of colon  
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18 6 cancer.

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20 7 **Word count:249**

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6 2 Merkel cell carcinoma is a rare skin cancer that originates from  
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9 3 neuroendocrine cells. This pathological condition has been related  
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11 4 recently to infection with a novel human polyomavirus, the Merkel cell  
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13 5 polyomavirus [Feng et al., 2008; Zur Hausen, 2008; Becker et al., 2009].  
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16 6 Like SV40 [Vilchez and Butel, 2004], Merkel cell polyomavirus appears  
17  
18 7 to play a causal role in tumorigenesis. Tumour induction and expansion is  
19  
20 8 preceded by a monoclonal integration of the virus into target cells.  
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22 9 Nonsense mutations or deletions in the early genomic region lead to the  
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24 10 expression of truncated large T antigen, loss of viral DNA replication,  
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26 11 and cell transformation [Shuda et al., 2008].  
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29 12 The assumed oncogenic power of the Merkel cell polyomavirus has  
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31 13 prompted research to explore its role in several human cancers, namely in  
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33 14 non-Merkel skin tumours, in neuroblastoma and other malignancies of the  
34  
35 15 central nervous system, in lung cancer, and mesothelioma. A series of  
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37 16 high-grade neuroendocrine tumours from different body sites was also  
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39 17 investigated, with negative results [Duncavage et al., 2009; Gandhi et al.,  
40  
41 18 2009; Sastre-Garau et al., 2009].  
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44 19 Other polyomaviruses, namely JCV and SV40, have been linked  
45  
46 20 previously to colon cancer [Markovics et al., 2005; Campello et al., 2010;  
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48 21 Gjoerup and Chang, 2010]. The hypothesis that polyomaviruses and  
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50 22 gastrointestinal malignancies are associated relies on several reports in  
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1 which sequences of JCV were detected in cancerous tissue, with different  
2 rates of prevalence at distinct times and in different geographic areas. In  
3 addition, a number of pathogenetic steps in the tumorigenesis associated  
4 with JCV have been described [White and Khalili, 2004]. A further  
5 rationale for the investigation of Merkel cell polyomavirus in colon  
6 cancer is that its epidemiological pattern is similar to that of JCV. It has  
7 been demonstrated recently that DNA sequences of both Merkel cell  
8 polyomavirus and JCV can be detected in sewage and environmental  
9 samples at high concentrations, thus supporting the faecal–oral route of  
10 their transmission [Boffil-Mas et al., 2010; Loyo et al., 2010].

11 Investigations of the possible association between Merkel cell  
12 polyomavirus and colon cancer are rare. Studies have been carried out  
13 solely on formalin-fixed paraffin-embedded archival tissues, and never  
14 compared with samples from control subjects enrolled concurrently  
15 [Kassem et al., 2009; Loyo et al., 2010]. In addition, the presence of  
16 Merkel cell polyomavirus in precancerous lesions of the colon has never  
17 been explored.

18 To address these issues, a prospective molecular case–control study was  
19 undertaken to detect the Merkel cell polyomavirus in patients with colon  
20 cancer and in their relatives, who were used as controls.

## 21 22 **Materials and Methods**

## 1 Patients and samples

2 In the period 2006–2008, patients with colon cancer and control subjects  
3 were enrolled consecutively at the Teaching Hospital of Verona, Italy.

4 This study was part of a large screening programme for colon–rectal  
5 cancer in the first-degree relatives of any cancer patient newly identified.

6 After informed consent for participation in the principal study had been  
7 obtained, colonoscopy was performed under conscious sedation. Among

8 all the relatives screened, the patients in whom a ‘large-biopsy’ specimen  
9 was taken from the colonic mucosa were enrolled because of hyperplastic

10 lesions, inflammation or any other suspicious condition. Specific

11 informed consent was obtained from each participant for the virological

12 survey undertaken on cancerous tissue or colon biopsies in addition to the

13 histological evaluation. Both the main screening study and this molecular

14 case–control study were approved by the Independent Ethics Committee.

15 The cases were 64 patients (40 male) with colon cancer whose histiotypes  
16 are reported in Table I. The mean age was 69.9 years, with a SD=11.0.

17 The controls were 80 relatives (43 male; 55 son/daughter, 23  
18 brother/sister and 2 parents) with a mean age of 53.7 years, SD=8.6.

19 According to the pathology results, adenoma or hyperplastic polyps were  
20 detected in 15 (18.8%) and 9 (11.2%) of the controls, respectively (see

21 Table I). The gender distribution was similar in both groups, while the

22 mean age, as expected, was significantly lower in controls ( $p=0.001$ ). In

1 addition to fresh tissue samples, a blood sample was also taken from each  
2 patient and control. All samples were stored at -80°C until analysed. A  
3 total of 288 specimens were examined.

#### 4 **Molecular evaluation**

5 The DNA was extracted with a commercial kit (Qiagen, Milan, Italy) and  
6 tested for suitability for PCR by amplifying the RNase P gene, as  
7 described recently [Comar et al., 2010]. The presence of Merkel cell  
8 polyomavirus DNA was detected using a conventional PCR. Specific  
9 primer pairs were designed to detect the viral Large T protein (LT3) and  
10 the major capsid protein (VP1) of Merkel cell polyomavirus, as described  
11 previously [Feng et al., 2008]. Positive controls cloned in plasmid vectors  
12 (pcDNA.MCV350) and negative DNA samples were included in each  
13 amplification run. Precautions were taken to avoid cross-contamination of  
14 the samples by means of unidirectional molecular workflow.  
15 Amplification products were sequenced with the Big Dye Terminator  
16 Cycle Sequencing Kit v.3.1 and analysed using an ABI Prism 310  
17 Genetic Analyzer (Applied Biosystems, Milan, Italy). Contiguous  
18 sequences were assembled using the Sequencer 4.5 software (Gene Codes  
19 Corp., Ann Arbor, MI). The sequences obtained were matched against  
20 those available from GenBank using the BLAST algorithm. The  
21 sequences were deposited in GenBank with the accession numbers  
22 HQ262569 and HQ262570.

## 1 **Statistics**

2 The sample size was estimated at 60 cases and 60 controls, assuming a  
3 prevalence of infection of 15% in controls, an OR=2,  $\alpha$  error=0.05, and  $\beta$   
4 error=0.20. Chi-square or Fisher exact tests were used to assess  
5 differences in frequency, and the Student t-test to evaluate differences in  
6 means. A logistic regression model was used to estimate the relationship  
7 between virus positivity and colon cancer, with adjustment for age.

8

## 9 **Results**

10 Overall, 11 sequences of Merkel cell polyomavirus, encompassing both  
11 regions tested, were detected in samples of colon tissue. No blood sample  
12 tested positive in either the cases or controls. The distribution of positive  
13 results by group and according to pathological features is reported in  
14 Table I. The rate of prevalence was 6.3% in the cases and 8.8% in the  
15 controls. This difference was not statistically significant in the univariate  
16 analysis. In addition, the rates of prevalence for preneoplastic lesions,  
17 namely adenomas and polyps, were 18.8% and 11.2%, respectively.  
18 When adjusted for age, no significant association was found between the  
19 rate of prevalence in cases and controls (prevalence of Merkel cell  
20 polyomavirus in colon cancer *vs.* prevalence in controls: OR=4.47;  
21 95%CI=0.61–28.66;  $p=0.147$ ). Furthermore, no significant difference was  
22 recorded when cancer was compared with preneoplastic disorders

1 (OR=0.53; 95%CI=0.11–2.57; p=0.426), and when preneoplastic tissues  
2 were compared with normal tissues (OR=1.71; 95%CI=0.35–8.34;  
3 p=0.503). Interestingly, the sole neuroendocrine tumour discovered did  
4 not harbour Merkel cell polyomavirus.

5 The amplification products of regions LT3 and VP1 were analysed by  
6 sequencing to confirm their identification and to assess sequence  
7 variability. The sequences of the conserved LT3 region were identical to  
8 almost all those published, and in particular to sequences of strains  
9 isolated in France (not shown). Two representative sequence patterns of  
10 the variable region VP1 are reported in Table II, along with the sequences  
11 of prototype strains and of other isolates retrieved from GenBank. The  
12 strain MCV-ITA61 was identical to the isolate MKT-31 and to other  
13 sequences discovered in France and Australia (GenBank accession  
14 numbers FM864211, AM992902, FJ009188/9). The strain MCV-ITA71  
15 was identical to MKT23 and to some American and European sequences  
16 (GenBank accession numbers FJ392560, FJ649206, AM992904/5). The  
17 level of identity of isolates in this survey was higher than 99% with the  
18 reference strain MCC350. Overall, the Merkel cell polyomavirus  
19 sequence patterns obtained in this study reproduced those of the  
20 genotypes that are circulating worldwide, and especially in France.

## 22 Discussion

1 The molecular case–control study reported herein was the first to focus on  
2 the role of Merkel cell polyomavirus in incident cases of colon cancer  
3 enrolled consecutively. Tissue samples were deep-frozen to ensure the  
4 preservation of viral components, because the detection of polyomavirus  
5 DNA may be impaired when archival fixed samples are tested [Touzé et  
6 al., 2009]. Similarly, the consecutive enrolment of controls was  
7 conducted from a cohort of subjects who underwent colonoscopy for  
8 cancer screening because they were the first-degree relatives of each  
9 patient. The enrolment of controls from relatives of the cases allowed  
10 correction for two exposure factors that could have been operating. First,  
11 past or present exposures to environmental determinants could have been,  
12 at least in part, common to the cases and controls. Second, genetic  
13 determinants of colon cancer could have been distributed homogeneously  
14 in the cases and their relatives. Hence, the control of both factors may  
15 have contributed to the disclosure of the putative role of the oncogenic  
16 polyomavirus.

17 In this study, Merkel cell polyomavirus DNA was detected in patients  
18 with colon cancer, but the rate of prevalence did not differ significantly  
19 from that in the well-structured control group. In addition, no Merkel cell  
20 polyomavirus sequence was detected in blood samples, thus excluding  
21 possible systemic involvement sustained by infection of PBMCs [Shuda  
22 et al., 2009].

1 The rates of prevalence that are reported herein were compared with those  
2 found in other surveys, in particular with those obtained using standard  
3 PCR to assess or confirm the presence of polyomavirus DNA by  
4 amplifying the same regions [Feng et al., 2008; Bialasiewicz et al., 2009,  
5 Goh et al., 2009; Wieland et al., 2009]. The findings of this survey were  
6 very similar to those of Feng et al. [2008], who reported a rate of  
7 prevalence of 8% in selected tissues from the gastro-intestinal tract, with  
8 the exclusion of tumours [Feng et al., 2008]. Conversely, in an archival  
9 series of cases of colon adenocarcinoma, no Merkel cell polyomavirus  
10 was detected [Sastre-Garau et al., 2009]. In a small retrospective case-  
11 control study, the rates of prevalence that were found by means of real-  
12 time PCR were 16% in specimens of colon cancer and 20% in control  
13 tissues. The study also demonstrated that the viral load was high in the  
14 polyomavirus-related cancer (i.e. Merkel cell carcinoma), whereas it was  
15 very low in tissues from other sites, for example, the urogenital tract.  
16 Tissues from the digestive tract had an intermediate viral load [Loyo et  
17 al., 2010]. Thus, the sensitivity of a standard PCR appears to be  
18 satisfactory when specimens of colon cancer, precancerous lesions and  
19 normal mucosa are compared, and in particular when fresh tissues are  
20 investigated. Collectively, these findings do not support the hypothesis  
21 that there is an aetiopathogenetic relationship between Merkel cell  
22 polyomavirus and colon cancer. The results point towards a simple

1 involvement of the gastrointestinal tract through the faecal–oral route of  
2 transmission of the virus [Feng et al., 2008; Gandhi et al., 2009; Loyo et  
3 al., 2010].

4 This study involved a novel evaluation of precancerous tissues, namely  
5 adenomas and polyps, in which the rates of prevalence were similar to  
6 those of the other intestinal tissues examined. The meaning of these  
7 findings remains speculative. The recovery of Merkel cell polyomavirus  
8 sequences from tumoral, precancerous and normal tissues should be  
9 considered to represent the transient presence of an infectious agent  
10 transmitted by the faecal–oral route [Bofill-Mas et al., 2010].  
11 Nevertheless, the possibility of the genomic integration of Merkel cell  
12 polyomavirus in permissive precancerous cells could be considered, and  
13 its significance in the oncogenic process could then be established. In any  
14 case, the oncogenic mechanism of Merkel cell polyomavirus differs from  
15 that of other polyomaviruses, including JCV, whose detection in  
16 adenomas and polyps has been considered to be an important step in the  
17 “hit-and-run” theory of tumorigenesis [Jung et al., 2008; Shuda et al.,  
18 2008].

19 In conclusion, the findings of this study are consistent with the hypothesis  
20 that the ubiquitous Merkel cell polyomavirus [Carter et al., 2009] is  
21 present fairly frequently in the gastrointestinal tract, and that its causal



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4 1 role in colon cancer, in addition to the role of other human and simian  
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6 2 polyomaviruses, should be reassessed.  
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