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Detection of SV40 in colon cancer: a molecular case-control study from North-East Italy

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Complete List of Authors:	Campello, Cesare; University of Trieste, Public Medicine Sc. Comar, Manola; Hygiene and Prevention Medicine, 1Department of Public Medicine Sciences, UCO Hygiene and Preventive Medicine, University of Trieste, and IRCCS "Burlo Garofolo", Trieste, Italy. Zanotta, Nunzia; Hygiene and Prevention Medicine, 1Department of Public Medicine Sciences, UCO Hygiene and Preventive Medicine, University of Trieste, Micozzi, Anna; University of Verona, Department of Anaesthesiology and Surgery Rodella, Luca; University of Verona, 4Department of Medicine and Public Health Poli, Albino; University of Verona, 4Department of Medicine and Public Health
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3 **Detection of SV40 in colon cancer: a molecular case-control study from**
4 **North-East Italy**
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9 Cesare Campello,¹ Manola Comar,¹ Nunzia Zanotta,¹ Anna Minicozzi,² Luca
10 Rodella,³ and Albino Poli⁴
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12

13 ¹Department of Public Medicine Sciences, University of Trieste, and IRCCS Burlo
14 Garofolo, Trieste, Italy
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16

17 ²Department of Anaesthesiology and Surgery, University of Verona, Italy
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20 ³General Surgery Unit, Endoscopy Service, Teaching Hospital of Verona, Italy
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23 ⁴Department of Medicine and Public Health, University of Verona, Italy
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28 **Corresponding Author.** Prof C. Campello, Department of Public Medicine
29 Sciences, University of Trieste, Via dell'Istria 65/1, 34137 Trieste, Italy. Phone
30 +39-040-773433, Fax +39-040-7600324; e-mail: campello@burlo.trieste.it.
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38 **Running head.** Detection of SV40 in colon cancer
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ABSTRACT

To explore the involvement of the simian polyomavirus SV40 in human colon cancer, a molecular case-control study was undertaken in patients and in their relatives living in an area where the spread of SV40 has already been documented. From 2006 to 2008, 94 colon cancer patients (age: 37-90 years) and 91 subjects (age: 32-70 years) relatives of each index case were enrolled. A blood sample and a specimen of cancer tissue or biopsy was collected, from each patient or control, respectively. Samples were analyzed twice for Polyomavirus (i.e. SV40, JCV and BKV) by PCR and by quantitative Real Time PCR (RT-qPCR) with reproducible results.

No BKV/JCV was detected either in normal or pathological tissues. SV40 was not present in control subjects, either normal tissue or in biopsies from adenomas or polyps. All blood samples were negative. Conversely, six adenocarcinoma specimens were positive for SV40 sequences (overall prevalence 6.4%, $p=0.03$ in comparison with controls). Nevertheless, the SV40-associated colon cancer risk proved statistically not significant ($OR=3.91$; $p=0.115$) when adjusted for age. Quantitation of SV40 DNA performed by RT-qPCR showed a low viral load ranging from 6.2×10^1 to 9×10^3 copies per reaction.

This molecular case-control survey showed, for the first time in fresh samples and by RT-q PCR, that SV40 can be detected in colon cancer tissue. However, the finding was not statistically significant when compared with a well-structured community control group. Thus, the role of SV40 and other polyomavirus in colon cancer genesis deserves further investigation.

Words count : 245

INTRODUCTION

Polyomaviruses are small, non-enveloped dsDNA viruses infecting a variety of vertebrate hosts, displaying an oncogenic power in experimental models and inducing tumorigenesis *in vivo*. Although most infections are asymptomatic, in immunocompromised hosts polyomaviruses cause a variety of disorders including cancer [Barbanti-Brodano et al., 2006]. The mechanism of cell transformation and tumoral induction is driven predominantly by the large T antigen (LT Ag) together with other oncoproteins, translating into an increased susceptibility of the target cell to DNA damage, genetic instability, and the expansion of transformed clones [White and Khalili, 2004].

Among polyomaviruses recognizing humans as the natural host, JCV has emerged as a notable candidate in the aetiology of a number of malignancies [Reiss and Khalili, 2003]. In immunocompromised patients, JCV acts as a neurotropic agent causing the progressive multifocal leucoencephalopathy and tumours of the central nervous system. JCV frequently displays kidney latency and urinary excretion, thus contaminating sewage and activating the faecal-oral route of transmission [Bofill-Mass and Girones, 2001]. Since the virus has been recovered from human gastro-intestinal tissues, a link with colon cancer has been suspected. This hypothesis relies on several reports in which, starting from the original observation of Laghi et al [Laghi et al., 1999], JCV sequences were detected in the colon cancer tissue and/or colon normal tissue with different rates of prevalence and throughout distinct times and areas. In addition, a number of molecular pathogenic steps in JCV colon tumorigenesis has been ascertained [Reiss and Khalili, 2003; White and Khalili, 2004]. However, some recent reports have tended to refuse any JCV relation with colorectal cancer [Newcomb et al., 2004; Lundstig et al., 2007; Giuliani et al., 2008; Militello et al., 2009].

The simian polyomavirus SV40 that entered accidentally the human population through contaminated poliomyelitis vaccines from 1958 to 1963 displays a powerful oncogenic activity. A robust relation has been established with the

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3 pleural mesothelioma, where asbestos exposure acts together with SV40 as a
4 cofactor to lower the asbestos threshold limit and to shorten the latency period of
5 carcinogenesis in hamsters [Kroczyńska et al., 2006]. Other malignancies
6 associated with SV40 have been recognized in the brain tumours, sarcomas and
7 non-Hodgkin lymphomas, while SV40 involvement in colon-rectal cancer has
8 been investigated poorly [Vilchez et al., 2004].

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10 A molecular case-control study was undertaken to explore the presence of the
11 SV40 in colon cancer patients in comparison with a cohort of their healthy
12 relatives living in an area of North-East Italy where the spread of SV40 has been
13 well documented [Comar et al., 2004; Comar et al., 2007].
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16 17 18 19 20 21 22 23 24 25 26 **MATERIALS AND METHODS**

27 28 **Patients, controls and samples**

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30 From 2006 to 2008, 94 patients with colon cancer (age: 37-90 years) and 91
31 control subjects (age: 32-70 years) were enrolled at the I Surgery Unit and
32 Endoscopy Service of the Teaching Hospital of Verona. This study was part of a
33 large screening programme for colon-rectal cancer in relatives of each cancer
34 index patient. The main demographic and pathological features of the cases and
35 controls are reported in tables I and II. Informed consent for the virological survey
36 undertaken in cancer tissue or colon biopsies, in addition to histological
37 evaluations, was obtained from each participant. The principal study and this
38 molecular epidemiological case-control study were approved by the local
39 Independent Ethical Committee. From each patient or control subject, a sample of
40 cancer tissue or a biopsy were collected, along with an EDTA blood sample.
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42 Blood cells and tissue samples were stored at -80°C until examination. The
43 histopathological assessment was performed at the Pathology Unit at the Medical
44 School of Verona.
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3 In addition, as a further control of polyomavirus detection, 67 urine samples (30
4 from healthy subjects of the same area and 37 from the cohort of cases/controls)
5 were collected consecutively and tested.
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10 11 **Molecular evaluation**

12 DNA was extracted with a commercial kit (Qiagen Roche Applied Science,
13 Mannheim, Germany). The DNA extraction was followed by the molecular
14 investigation in a laboratory with appropriated facilities to control cross-
15 contamination.
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21 Each DNA sample was first assessed for PCR suitability by amplifying β -globin
22 gene sequences, as previously described [Comar et al., 2007]. PCR amplification
23 of PYV (BKV, JCV, and SV40) region was carried out with the set of primers
24 PYV.F (5'-TAGGTGCCAACCTATGGAACAGA-3') and PYV.R (5'-
25 GAAAGTCTTTAGGGTCTTCTACC-3') which amplify a well-conserved Tag
26 amino-terminal sequence common to polyomavirus. PCR conditions were as
27 follow: 1 min at 94°C, 30 sec at 55°C and 40 sec at 72°C, for 40 cycles. JCV
28 Mad1, BKV Dunlop, and SV40-B2E DNA were included as positive controls. The
29 samples, along with negative and positive controls, were blindly analyzed twice.
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40 In order to enhance the sensitivity of detection, all samples were analyzed for
41 SV40, JCV and BKV by a Real Time quantitative polymerase chain reaction (RT-
42 qPCR) using the Applied Biosystems 7500 Real Time PCR System (Applied
43 Biosystems, Milan, Italy). In short, 10 μ l of DNA sample, and 10 μ l of each
44 specific standard scale dilution, from 10 to 10⁷ copies, were added to a final
45 volume of 50 μ l of reaction-mix in duplicate as previously described [Comar et al.,
46 2007]. The single-copy cellular RNase P gene (Applied Biosystems, Milan, Italy)
47 was used to normalize viral copies to the number of cell equivalents. In addition,
48 detected amplicons were subjected to a sequencing reaction using Big Dye
49 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Milan,
50 Italy). PCR products were analysed by ABI 310 Genetic analyser (Applied
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3 Biosystems, Milan, Italy). The assemblage of sequences was carried out by the
4 Sequencer package 4.5 of Gene Codes Corporation and analyzed with the BLAST
5 Software.
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10 11 **Statistical analysis**

12 Data are presented as frequency, proportion or mean, as appropriate. The Chi
13 square and Fisher exact tests were used to test differences in frequencies and the
14 Student-t test to assess differences in mean. Exact Logistic Regression Model was
15 used to estimate the association between SV40 infection and colon cancer,
16 adjusted for age. Median Unbiased Estimation (MUE) without Mid-p test was
17 used to test the statistical significance. The differences were considered significant
18 when $P < 0.05$.
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28 **RESULTS**

29 Table I shows the main demographic characteristic of cases and controls. The
30 gender distribution was similar in both groups, while the mean age, as expected,
31 was significantly lower in controls ($p = 0.001$). Fifty seven controls (62.6%) had no
32 colorectal lesion. The most frequent lesions were adenomas (58.8% of lesions) or
33 hyperplastic polyps (29.4% of lesions). Among the cases, the most frequent cancer
34 was adenocarcinoma (90.4%) (Table II).
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43 BKV and JCV were not detected either in normal or pathological tissues. SV40
44 was absent in specimens from control subjects, either in normal tissue or in
45 biopsies of adenomas and polyps. Conversely, six adenocarcinoma specimens
46 tested positive for SV40 LT sequence, leading to an overall prevalence of 6.4%.
47 Quantitation of SV40 DNA showed a low viral load ranging from 6.2×10^1 to $9 \times$
48 10^3 copies per reaction with a geometric mean as 6.4×10^2 copies (data not
49 shown). Results obtained from analysis of each sample were reproducible by both
50 molecular methods used (*i.e.* PCR and RT-qPCR).
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59 In the univariate analysis, SV40 frequencies in cases and controls were
60 significantly different (Fisher exact test, $p = 0.03$). Since the age of the two groups

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3 differed, the association between colon cancer and SV40 positivity was tested by
4 an exact logistic regression, adjusted for age. As reported in Table III, the colon
5 cancer risk increased by about 13% with each year of aging (OR=1.134;
6 P=0.0001), while the OR of SV40-associated colon cancer risk resulted to be
7 statistically not significant (OR=3.91; p=0.115).
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10 Finally, owing to the unusual absence of human polyomavirus in cancer tissues
11 here examined, a survey was undertaken to control the polyomavirus shedding in
12 urine of subjects from this area. While no BKV and SV40 virus was detected, JCV
13 was frequently found (25/67 samples, 37%). In particular, 5/5 patients, 14/32
14 controls (43.7%) and 6/30 pregnant women (20%) were JCV positive.
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17 18 19 20 21 22 23 24 **DISCUSSION**

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26 This molecular case-control study showed that sequences of polyomavirus SV40,
27 but not that of JCV or BKV, were detectable in patients suffering with colon
28 cancer. These results were attained by means of a methodological approach
29 worthy of comment for a number of reasons. Incident cases were enrolled
30 consecutively and cancer samples were immediately deep-frozen until
31 examination, thus preserving viral components. Also the enrolment of controls
32 was performed, consecutively, from a cohort of subjects undergoing colonoscopy
33 for cancer screening because they were first grade relatives of each patient. Thus,
34 any previous and/or present exposure common to cases and controls could
35 reinforce the possible link of distinct polyomavirus with cancer tissue. In addition,
36 in the comparison between relatives the influence of common genetic
37 determinants of tumorigenesis might favour the emergence of a putative viral role.
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39 Finally, polyomavirus footprints were explored by means of a sensitive
40 quantitative real-time PCR, thus assuring the characteristics of accuracy and
41 specificity that are recommended in this type of investigation [Shah, 2006].
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45 Although the lack of BKV was expected according to published reports, the
46 absence of JCV was a rather surprising finding. High prevalence rates of JCV
47 infecting colon cancer tissues have been reported repeatedly in various
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3 geographical areas and some pathogenetic steps have been offered. However,
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5 conflicting results on the role of JCV in colon tumorigenesis are emerging and a
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7 possible technical weakness was suspected [Shah, 2006; Militello et al., 2009]. In
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9 this survey, a possible low sensitivity on JCV recovery was ruled out when viral
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11 kidney latency was explored and urinary excretion was documented either in cases
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13 or in controls.
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16 The rationale behind investigating the role of SV40 in colon cancer lies in three
17
18 main issues. First, the oncogenic mechanism of the simian virus is similar to that
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20 of JCV, as proven by the detection of the LT antigen expressed in JCV transfected
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22 cells similar to SV40 oncoprotein [Vilchez et al., 2004 Barbanti-Brodano et al.,
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24 2006]. Secondly, under strict experimental conditions, SV40 proved to be a strong
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26 transforming agent of adenomatous cell line predisposed genetically to colon
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28 cancer [Kopelovich, 1984]. In addition, transgenic mice developed intestinal
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30 dysplasia due to the binding of the SV40 oncoprotein to pRb protein family
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32 [Markovics et al., 2005]. Finally, SV40 transmission by faecal-oral route has been
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34 well ascertained. SV40 may establish kidney latency with intermittent viral
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36 shedding both in simians and humans [Vanchiere et al., 2005; Thomas et al.,
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38 2007]. Accordingly, the virus could be recovered from sewage and transmission
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40 from simian to human could take place [Engels et al., 2004].
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43 Although SV40 was detected in patients, its relation with cancer was statistically
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45 not significant when compared to a well structured community control group. In
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47 general, the low detection rate of simian polyomavirus was consistent with recent
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49 seroepidemiologic data, showing the prevalence of SV40 antibody without cross-
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51 reactivity with human polyomavirus BKV/JCV is about 2%. [Kean et al.,2009]. In
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53 addition, the findings reported above seem to indicate a simple involvement of
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55 cancer tissue, possibly favoured by the underlying tumoral disease, without any
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57 aetio-pathogenic significance. This result emerged when a multilogistic analysis,
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59 adjusted for age, removed the putative association between SV40 infection and
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colon cancer arising from the univariate analysis. Thus stringent conditions for

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3 molecular assessment, suitable control groups and a thorough statistical evaluation
4 are needed in this type of survey before establishing a causal relationship.
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7 The 'hit and run' theory of polyomavirus oncogenesis, particularly for JCV, has
8 been advanced in colon cancer genesis [Jung et al., 2008]. Accordingly, after the
9 oncoprotein-mediated induction of genetic instability and promotion of malignant
10 transformation, tumour growth translates into the loss of both LT sequences and
11 oncoprotein itself. Hence, it has been suggested that polyomavirus DNA should be
12 sought, preferentially in adenomas and in low-stage cancer tissues. In this survey,
13 while genomic sequences could still be detected in cancerous samples, no LT
14 antigen sequence was present in precancerous lesions discovered during the
15 screening of control subjects. At the same time, the low viral load measured here
16 might be insufficient for the oncoprotein expression [Jung et al., 2008]. Two novel
17 mechanisms recently described support the oncogenic power of a low number of
18 infecting SV40, namely antisense transcript production preserving the episomal
19 SV40 cellular lysis and the activation of IGF-I pathway sustained by the Tag-p53
20 complex. However, these mechanisms have only been demonstrated *in vitro* and in
21 the mesothelial cell model [Carbone et al., 2008; Bocchetta et al., 2008].
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24 In conclusion, this molecular case-control study from North-East Italy showed, for
25 the first time in fresh samples and by quantitative Real-Time PCR, that SV40
26 could be detected in colon cancer tissue. However, the findings were not
27 statistically significant when compared to a well-structured community control
28 group. Thus, the role of SV40 and other polyomavirus in colon cancer genesis
29 deserves further investigation.
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42 **Ethics approval:** ethics approval was obtained
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For Peer Review

Table I – Main demographic characteristics of cancer patients and controls subjects.

Variable	controls		cases		p
	91		94		
	n.	(%)	n.	(%)	
<u>Gender</u>					
Males	49	(53.9)	60	(63.3)	0.151
Females	42	(46.1)	34	(36.7)	
<u>Age</u>					
mean (DS)	54.1	(8.8)	69.1	(12.5)	<0.001
<u>Relative to index case</u>					
Son/daughter	60	(66.7)	--		
Brother/sister	28	(31.1)	--		
Father/mother	3	(2.2)	--		

Table II – Main pathology features of the cases and controls, and SV40 positivity

	n.	%	SV40 positivity	
			n. (%)	(95%CI)
CONTROLS				
No lesion	57	(62.6)	0	--
adenoma	20	(22.0)	0	--
Hyperplastic polyp	10	(11.0)	0	--
Other conditions	4	(4.4)	0	--
Total	91	--	0*	(0.0 – 3.2)
CASES				
Adenocarcinoma	85	(90.4)	6 (7.1)	(2.9 – 14.1)
Mucinous adenocarcinoma	8	(8.5)	0	--
Neuroendocrine tumor	1	(1.1)	0	--
Total	94	--	6* (6.4)	(2.6 – 12.8)

*Fisher exact test: P=0.031

Table III – Age-adjusted Odds Ratio for SV40-related colon cancer
(exact logistic regression analysis).

Variable	Odds Ratio	(95% CI)	p
Age	1.134	1.092 – 1.178	<0.0001
SV40 (neg. as reference)	3.91	0.000 – $+\infty$	0.115

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