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Cesare Campello, Manola Comar, Nunzia Zanotta, Anna Micozzi, Luca Rodella, et al.. Detection of SV40 in colon cancer: a molecular case-control study from North-East Italy. *Journal of Medical Virology*, 2010, 82 (7), pp.1197. 10.1002/jmv.21798 . hal-00552411

HAL Id: hal-00552411

<https://hal.science/hal-00552411>

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Journal:	<i>Journal of Medical Virology</i>
Manuscript ID:	JMV-09-1671.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	05-Feb-2010
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Keywords:	human polyomavirus , colon cancer, case-control study

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

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Running head. Detection of SV40 in colon cancer

Key words. SV40, human polyomavirus; colon cancer; case-control study

Word count: 2082

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

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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
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5 cofactor to lower the asbestos threshold limit and to shorten the latency period of
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7 carcinogenesis in hamsters [Kroczyńska et al., 2006]. Other malignancies
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9 associated with SV40 have been recognized in the brain tumours, sarcomas and
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11 non-Hodgkin lymphomas, while SV40 involvement in colon-rectal cancer has
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13 been investigated poorly [Vilchez et al., 2004].
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16 A molecular case-control study was undertaken to explore the presence of the
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18 SV40 in colon cancer patients in comparison with a cohort of their healthy
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20 relatives living in an area of North-East Italy where the spread of SV40 has been
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22 well documented [Comar et al., 2004; Comar et al., 2007].
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25 26 **MATERIALS AND METHODS**

27 28 **Patients, controls and samples**

29
30 From 2006 to 2008, 94 patients with colon cancer (age: 37-90 years) and 91
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32 control subjects (age: 32-70 years) were enrolled at the I Surgery Unit and
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34 Endoscopy Service of the Teaching Hospital of Verona. This study was part of a
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36 large screening programme for colon-rectal cancer in relatives of each cancer
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38 index patient. The main demographic and pathological features of the cases and
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40 controls are reported in tables I and II. Informed consent for the virological survey
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42 undertaken in cancer tissue or colon biopsies, in addition to histological
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44 evaluations, was obtained from each participant. The principal study and this
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46 molecular epidemiological case-control study were approved by the local
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48 Independent Ethical Committee. From each patient or control subject, a sample of
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50 cancer tissue or a biopsy were collected, along with an EDTA blood sample.
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52 Blood cells and tissue samples were stored at -80°C until examination. The
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54 histopathological assessment was performed at the Pathology Unit at the Medical
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56 School of Verona.
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In addition, as a further control of polyomavirus detection, 67 urine samples (30 from healthy subjects of the same area and 37 from the cohort of cases/controls) were collected consecutively and tested.

Molecular evaluation

DNA was extracted with a commercial kit (Qiagen Roche Applied Science, Mannheim, Germany). The DNA extraction was followed by the molecular investigation in a laboratory with appropriated facilities to control cross-contamination.

Each DNA sample was first assessed for PCR suitability by amplifying β -globin gene sequences, as previously described [Comar et al., 2007]. PCR amplification of PYV (BKV, JCV, and SV40) region was carried out with the set of primers PYV.F (5'-TAGGTGCCAACCTATGGAACAGA-3') and PYV.R (5'-GAAAGTCTTTAGGGTCTTCTACC-3') which amplify a well-conserved Tag amino-terminal sequence common to polyomavirus. PCR conditions were as follow: 1 min at 94°C, 30 sec at 55°C and 40 sec at 72°C, for 40 cycles. JCV Mad1, BKV Dunlop, and SV40-B2E DNA were included as positive controls. The samples, along with negative and positive controls, were blindly analyzed twice.

In order to enhance the sensitivity of detection, all samples were analyzed for SV40, JCV and BKV by a Real Time quantitative polymerase chain reaction (RT-qPCR) using the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Milan, Italy). In short, 10 μ l of DNA sample, and 10 μ l of each specific standard scale dilution, from 10 to 10⁷ copies, were added to a final volume of 50 μ l of reaction-mix in duplicate as previously described [Comar et al., 2007].The single-copy cellular RNase P gene (Applied Biosystems, Milan, Italy) was used to normalize viral copies to the number of cell equivalents. In addition, detected amplicons were subjected to a sequencing reaction using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Milan, Italy). PCR products were analysed by ABI 310 Genetic analyser (Applied

Biosystems, Milan, Italy). The assemblage of sequences was carried out by the Sequencer package 4.5 of Gene Codes Corporation and analyzed with the BLAST Software.

Statistical analysis

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

RESULTS

Table I shows the main demographic characteristic of cases and controls. The gender distribution was similar in both groups, while the mean age, as expected, was significantly lower in controls ($p = 0.001$). Fifty seven controls (62.6%) had no colorectal lesion. The most frequent lesions were adenomas (58.8% of lesions) or hyperplastic polyps (29.4% of lesions). Among the cases, the most frequent cancer was adenocarcinoma (90.4%) (Table II).

BKV and JCV were not detected either in normal or pathological tissues. SV40 was absent in specimens from control subjects, either in normal tissue or in biopsies of adenomas and polyps. Conversely, six adenocarcinoma specimens tested positive for SV40 LT sequence, leading to an overall prevalence of 6.4%. Quantitation of SV40 DNA showed a low viral load ranging from 6.2×10^1 to 9×10^3 copies per reaction with a geometric mean as 6.4×10^2 copies (data not shown). Results obtained from analysis of each sample were reproducible by both molecular methods used (*i.e.* PCR and RT-qPCR).

In the univariate analysis, SV40 frequencies in cases and controls were significantly different (Fisher exact test, $p = 0.03$). Since the age of the two groups

differed, the association between colon cancer and SV40 positivity was tested by an exact logistic regression, adjusted for age. As reported in Table III, the colon cancer risk increased by about 13% with each year of aging (OR=1.134; P=0.0001), while the OR of SV40-associated colon cancer risk resulted to be statistically not significant (OR=3.91; p=0.115).

Finally, owing to the unusual absence of human polyomavirus in cancer tissues here examined, a survey was undertaken to control the polyomavirus shedding in urine of subjects from this area. While no BKV and SV40 virus was detected, JCV was frequently found (25/67 samples, 37%). In particular, 5/5 patients, 14/32 controls (43.7%) and 6/30 pregnant women (20%) were JCV positive.

DISCUSSION

This molecular case-control study showed that sequences of polyomavirus SV40, but not that of JCV or BKV, were detectable in patients suffering with colon cancer. These results were attained by means of a methodological approach worthy of comment for a number of reasons. Incident cases were enrolled consecutively and cancer samples were immediately deep-frozen until examination, thus preserving viral components. Also the enrolment of controls was performed, consecutively, from a cohort of subjects undergoing colonoscopy for cancer screening because they were first grade relatives of each patient. Thus, any previous and/or present exposure common to cases and controls could reinforce the possible link of distinct polyomavirus with cancer tissue. In addition, in the comparison between relatives the influence of common genetic determinants of tumorigenesis might favour the emergence of a putative viral role. Finally, polyomavirus footprints were explored by means of a sensitive quantitative real-time PCR, thus assuring the characteristics of accuracy and specificity that are recommended in this type of investigation [Shah, 2006].

Although the lack of BKV was expected according to published reports, the absence of JCV was a rather surprising finding. High prevalence rates of JCV infecting colon cancer tissues have been reported repeatedly in various

geographical areas and some pathogenetic steps have been offered. However, conflicting results on the role of JCV in colon tumorigenesis are emerging and a possible technical weakness was suspected [Shah, 2006; Militello et al., 2009]. In this survey, a possible low sensitivity on JCV recovery was ruled out when viral kidney latency was explored and urinary excretion was documented either in cases or in controls.

The rationale behind investigating the role of SV40 in colon cancer lies in three main issues. First, the oncogenic mechanism of the simian virus is similar to that of JCV, as proven by the detection of the LT antigen expressed in JCV transfected cells similar to SV40 oncoprotein [Vilchez et al., 2004; Barbanti-Brodano et al., 2006]. Secondly, under strict experimental conditions, SV40 proved to be a strong transforming agent of adenomatous cell line predisposed genetically to colon cancer [Kopelovich, 1984]. In addition, transgenic mice developed intestinal dysplasia due to the binding of the SV40 oncoprotein to pRb protein family [Markovics et al., 2005]. Finally, SV40 transmission by faecal-oral route has been well ascertained. SV40 may establish kidney latency with intermittent viral shedding both in simians and humans [Vanchiere et al., 2005; Thomas et al., 2007]. Accordingly, the virus could be recovered from sewage and transmission from simian to human could take place [Engels et al., 2004].

Although SV40 was detected in patients, its relation with cancer was statistically not significant when compared to a well structured community control group. In general, the low detection rate of simian polyomavirus was consistent with recent seroepidemiologic data, showing the prevalence of SV40 antibody without cross-reactivity with human polyomavirus BKV/JCV is about 2%. [Kean et al., 2009]. In addition, the findings reported above seem to indicate a simple involvement of cancer tissue, possibly favoured by the underlying tumoral disease, without any aetio-pathogenic significance. This result emerged when a multilogistic analysis, adjusted for age, removed the putative association between SV40 infection and colon cancer arising from the univariate analysis. Thus stringent conditions for

molecular assessment, suitable control groups and a thorough statistical evaluation are needed in this type of survey before establishing a causal relationship.

The ‘hit and run’ theory of polyomavirus oncogenesis, particularly for JCV, has been advanced in colon cancer genesis [Jung et al., 2008]. Accordingly, after the oncoprotein-mediated induction of genetic instability and promotion of malignant transformation, tumour growth translates into the loss of both LT sequences and oncoprotein itself. Hence, it has been suggested that polyomavirus DNA should be sought, preferentially in adenomas and in low-stage cancer tissues. In this survey, while genomic sequences could still be detected in cancerous samples, no LT antigen sequence was present in precancerous lesions discovered during the screening of control subjects. At the same time, the low viral load measured here might be insufficient for the oncoprotein expression [Jung et al., 2008]. Two novel mechanisms recently described support the oncogenic power of a low number of infecting SV40, namely antisense transcript production preserving the episomal SV40 cellular lysis and the activation of IGF-I pathway sustained by the Tag-p53 complex. However, these mechanisms have only been demonstrated *in vitro* and in the mesothelial cell model [Carbone et al., 2008; Bocchetta et al., 2008].

In conclusion, this molecular case-control study from North-East Italy showed, for the first time in fresh samples and by quantitative Real-Time PCR, that SV40 could be detected in colon cancer tissue. However, the findings were not statistically significant when compared to a well-structured community control group. Thus, the role of SV40 and other polyomavirus in colon cancer genesis deserves further investigation.

Acknowledgments. The technical assistance of Mrs. Morena Nicolis is grateful acknowledged.

Funding: PRIN 2007, Ministry for University and Research, Italy

Competing interest: none

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

			SV40 positivity	
	n.	%	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