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

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

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| Keywords:            | Merkel cell polyomavirus, colon cancer, molecular case–control study  |
Table I. Main pathological features of the cases and controls, and positive results for Merkel cell polyomavirus

<table>
<thead>
<tr>
<th></th>
<th>CONTROLS</th>
<th>CASES</th>
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<tbody>
<tr>
<td></td>
<td>n.</td>
<td>%</td>
</tr>
<tr>
<td><strong>MCPyV positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No lesion</td>
<td>52</td>
<td>65.0</td>
</tr>
<tr>
<td>Adenoma</td>
<td>15</td>
<td>18.8</td>
</tr>
<tr>
<td>Hyperplastic polyp</td>
<td>9</td>
<td>11.2</td>
</tr>
<tr>
<td>Other conditions</td>
<td>4</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>80</td>
<td>--</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>GenBank Accession N.</th>
<th>VP1 gene - Nucleotide position</th>
<th>Country</th>
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</thead>
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<tr>
<td>MCC350</td>
<td>EU375803</td>
<td>T C C T</td>
<td>USA</td>
</tr>
<tr>
<td>MCC339</td>
<td>EU375804</td>
<td>C G T A</td>
<td>USA</td>
</tr>
<tr>
<td>MKT-23</td>
<td>FM864208</td>
<td>C G C T</td>
<td>France</td>
</tr>
<tr>
<td>MKT-31</td>
<td>FM864210</td>
<td>C C C T</td>
<td>France</td>
</tr>
<tr>
<td>VP1-Mpt2</td>
<td>AM992903</td>
<td>C G C A</td>
<td>France</td>
</tr>
<tr>
<td>MCV ITA 61</td>
<td>HQ262570</td>
<td>C C C T</td>
<td>Italy</td>
</tr>
<tr>
<td>MCV ITA 71</td>
<td>HQ262569</td>
<td>C G C T</td>
<td>Italy</td>
</tr>
</tbody>
</table>
A MOLECULAR CASE–CONTROL STUDY ON THE MERKEL CELL POLYOMAVIRUS IN COLON CANCER

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Running title. Merkel polyomavirus and colon cancer

Key words. Merkel cell polyomavirus; colon cancer; molecular case–control study

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Word count: 1945
Abstract

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

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

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

Sequence analysis of the viral LT3 and VP1 regions showed high homology (>99%) with those of strains circulating worldwide, especially with genotypes detected in France.
The findings of this survey are consistent with the hypothesis that the Merkel cell polyomavirus, in addition to other human polyomaviruses, can be recovered frequently from the gastrointestinal tract, because it is transmitted throughout the faecal–oral route. Moreover, the study does not indicate a role for Merkel cell polyomavirus in the genesis of colon cancer.

**Word count: 249**
Merkel cell carcinoma is a rare skin cancer that originates from neuroendocrine cells. This pathological condition has been related recently to infection with a novel human polyomavirus, the Merkel cell polyomavirus [Feng et al., 2008; Zur Hausen, 2008; Becker et al., 2009]. Like SV40 [Vilchez and Butel, 2004], Merkel cell polyomavirus appears to play a causal role in tumorigenesis. Tumour induction and expansion is preceded by a monoclonal integration of the virus into target cells. Nonsense mutations or deletions in the early genomic region lead to the expression of truncated large T antigen, loss of viral DNA replication, and cell transformation [Shuda et al., 2008]. The assumed oncogenic power of the Merkel cell polyomavirus has prompted research to explore its role in several human cancers, namely in non-Merkel skin tumours, in neuroblastoma and other malignancies of the central nervous system, in lung cancer, and mesothelioma. A series of high-grade neuroendocrine tumours from different body sites was also investigated, with negative results [Duncavage et al., 2009; Gandhi et al., 2009; Sastre-Garau et al., 2009]. Other polyomaviruses, namely JCV and SV40, have been linked previously to colon cancer [Markovics et al., 2005; Campello et al., 2010; Gjoerup and Chang, 2010]. The hypothesis that polyomaviruses and gastrointestinal malignancies are associated relies on several reports in
which sequences of JCV were detected in cancerous tissue, with different
rates of prevalence at distinct times and in different geographic areas. In
addition, a number of pathogenetic steps in the tumorigenesis associated
with JCV have been described [White and Khalili, 2004]. A further
rationale for the investigation of Merkel cell polyomavirus in colon
cancer is that its epidemiological pattern is similar to that of JCV. It has
been demonstrated recently that DNA sequences of both Merkel cell
polyomavirus and JCV can be detected in sewage and environmental
samples at high concentrations, thus supporting the faecal–oral route of
their transmission [Boffil-Mas et al., 2010; Loyo et al., 2010].
Investigations of the possible association between Merkel cell
polyomavirus and colon cancer are rare. Studies have been carried out
solely on formalin-fixed paraffin-embedded archival tissues, and never
compared with samples from control subjects enrolled concurrently
[Kassem et al., 2009; Loyo et al., 2010]. In addition, the presence of
Merkel cell polyomavirus in precancerous lesions of the colon has never
been explored.
To address these issues, a prospective molecular case–control study was
undertaken to detect the Merkel cell polyomavirus in patients with colon
cancer and in their relatives, who were used as controls.

Materials and Methods
Patients and samples

In the period 2006–2008, patients with colon cancer and control subjects were enrolled consecutively at the Teaching Hospital of Verona, Italy. This study was part of a large screening programme for colon–rectal cancer in the first-degree relatives of any cancer patient newly identified. After informed consent for participation in the principal study had been obtained, colonoscopy was performed under conscious sedation. Among all the relatives screened, the patients in whom a ‘large-biopsy’ specimen was taken from the colonic mucosa were enrolled because of hyperplastic lesions, inflammation or any other suspicious condition. Specific informed consent was obtained from each participant for the virological survey undertaken on cancerous tissue or colon biopsies in addition to the histological evaluation. Both the main screening study and this molecular case–control study were approved by the Independent Ethics Committee.

The cases were 64 patients (40 male) with colon cancer whose histiotypes are reported in Table I. The mean age was 69.9 years, with a SD=11.0. The controls were 80 relatives (43 male; 55 son/daughter, 23 brother/sister and 2 parents) with a mean age of 53.7 years, SD=8.6. According to the pathology results, adenoma or hyperplastic polyps were detected in 15 (18.8%) and 9 (11.2%) of the controls, respectively (see Table I). The gender distribution was similar in both groups, while the mean age, as expected, was significantly lower in controls (p=0.001). In
addition to fresh tissue samples, a blood sample was also taken from each patient and control. All samples were stored at -80°C until analysed. A total of 288 specimens were examined.

Molecular evaluation

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

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

Results

Overall, 11 sequences of Merkel cell polyomavirus, encompassing both regions tested, were detected in samples of colon tissue. No blood sample tested positive in either the cases or controls. The distribution of positive results by group and according to pathological features is reported in Table I. The rate of prevalence was 6.3% in the cases and 8.8% in the controls. This difference was not statistically significant in the univariate analysis. In addition, the rates of prevalence for preneoplastic lesions, namely adenomas and polyps, were 18.8% and 11.2%, respectively. When adjusted for age, no significant association was found between the rate of prevalence in cases and controls (prevalence of Merkel cell polyomavirus in colon cancer vs. prevalence in controls: OR=4.47; 95%CI=0.61–28.66; p=0.147). Furthermore, no significant difference was recorded when cancer was compared with preneoplastic disorders.
(OR=0.53; 95%CI=0.11–2.57; p=0.426), and when preneoplastic tissues were compared with normal tissues (OR=1.71; 95%CI=0.35–8.34; p=0.503). Interestingly, the sole neuroendocrine tumour discovered did not harbour Merkel cell polyomavirus.

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

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

In this study, Merkel cell polyomavirus DNA was detected in patients with colon cancer, but the rate of prevalence did not differ significantly from that in the well-structured control group. In addition, no Merkel cell polyomavirus sequence was detected in blood samples, thus excluding possible systemic involvement sustained by infection of PBMCs [Shuda et al., 2009].
The rates of prevalence that are reported herein were compared with those found in other surveys, in particular with those obtained using standard PCR to assess or confirm the presence of polyomavirus DNA by amplifying the same regions [Feng et al., 2008; Bialasiewicz et al., 2009, Goh et al., 2009; Wieland et al., 2009]. The findings of this survey were very similar to those of Feng et al. [2008], who reported a rate of prevalence of 8% in selected tissues from the gastro-intestinal tract, with the exclusion of tumours [Feng et al., 2008]. Conversely, in an archival series of cases of colon adenocarcinoma, no Merkel cell polyomavirus was detected [Sastre-Garau et al., 2009]. In a small retrospective case–control study, the rates of prevalence that were found by means of real-time PCR were 16% in specimens of colon cancer and 20% in control tissues. The study also demonstrated that the viral load was high in the polyomavirus-related cancer (i.e. Merkel cell carcinoma), whereas it was very low in tissues from other sites, for example, the urogenital tract. Tissues from the digestive tract had an intermediate viral load [Loyo et al., 2010]. Thus, the sensitivity of a standard PCR appears to be satisfactory when specimens of colon cancer, precancerous lesions and normal mucosa are compared, and in particular when fresh tissues are investigated. Collectively, these findings do not support the hypothesis that there is an aetiopathogenetic relationship between Merkel cell polyomavirus and colon cancer. The results point towards a simple
involvement of the gastrointestinal tract through the faecal–oral route of transmission of the virus [Feng et al., 2008; Gandhi et al., 2009; Loyo et al., 2010].

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

In conclusion, the findings of this study are consistent with the hypothesis that the ubiquitous Merkel cell polyomavirus [Carter et al., 2009] is present fairly frequently in the gastrointestinal tract, and that its causal
role in colon cancer, in addition to the role of other human and simian polyomaviruses, should be reassessed.

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References


