APPLICATION OF REAL-TIME PCR TECHNOLOGIES FOR ANALYSIS OF JCV AS A HUMAN CANCEROGEN

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ABSTRACT
Viral agents such as Polyomavirus JC (JCV) raise an increased interest and are discussed as factors for development of several human malignancies. Real time PCR techniques allow better characterization of the studied samples as they possess superior sensitivity when compared to conventional PCR. Also quantitative analysis of the experimental results can be performed and information about the dynamics of the process is determined.

Real time PCR technologies were applied to investigate fresh frozen biopsies from 132 patients for two particular JCV genomic sequences (LT and NCCR) that might be related to malignant transformation. Patients were divided into groups according to their histological type, and included: glioblastoma patients (n=39); astrocytoma patients (n=19); oligodendrogliomas (n=12); colorectal cancer patients (n=44); and patients with polyps (n=18). Colon samples were paired with matched adjacent mucosa.

Results showed that viral genomic sequences were present in 22.7% to 61.5% among the different groups. Positive samples had a low starting viral number, as a late threshold cycle that varied from 36 to 49 was observed. The prevalence of the non-coding control region (NCCR) was lower than the prevalence of LT and only 12 of 70 brain samples were found positive. None of the tested premalignant colonic lesions and cancers was positive, whereas two normal tissues had viral load in the range of 10-1000 starting copies.

The disproportion between the results for LT and NCCR might be suggested to be either due to clonally integrated LT fragments, with loss of genetic material, or changes in the NCCR. This might lead to disturbance in the productive course of the infection and a premise for continuous interaction of viral regulatory proteins with cell molecules that are responsible for the control of the cell cycle may be establish. This subsequently might lead to malignant transformation. As infectious diseases acquire relevance in this process, new tools for cancer management and prevention are gained.


Keywords: real time PCR, JC polyomavirus, brain tumours, colorectal cancer, polyp, oncogenesis, malignant transformation

Introduction
Human cancerogenesis is a complex process where different risk factors, such as ionizing, non-ionizing radiation, various chemicals, dietary factors (1) and hereditary syndromes (20) are involved. However these factors account for a relatively small portion of all human tumours (3) and other agents should also be considered. Although the idea that viruses might cause the development of tumours is not new, current molecular studies are focused on changes in human DNA (2) such as: point mutations; hypomethylation of DNA, and subsequent gene activation; loss of genetic material from tumour-suppressor genes; loss of function associated with mutations in the p53 tumor-suppressor gene (13), etc. Infectious diseases, on the other hand, do not represent mainstream oncologic research but their importance as pathogenic elements in human cancer increases (21).

An example of an oncogenic virus is the human Polyomavirus hominis 2 (JCV), which has been identified in samples from primary brain tumours, gastrointestinal malignancies and pre-cancerous lesions. Nevertheless, the role of this infection has been a subject to controversial reports necessitating further studies (10).

The replicative strategy of JCV depends on induction of an S phase environment in the infected cells and stimulation of the cellular machinery for specific enzyme production, as the virus does not encode own replication enzymes. Host cells are manipulated in a complex manner, where the so called large T-antigen (LT) (7) is mainly involved. It can interact with tumour-suppressor proteins like p53 and pRb, leading to their inactivation (5) and stimulate signal pathways as it activates some proto-oncogenes such as beta-catenin and insulin receptor substrate 1 (5). Key points in the viral replication are controlled by the non-coding control region (NCCR) in the viral genome which makes it an essential factor for formation of mature virions (7).

A hypothesis was proposed that JCV infection may be an alternative to low frequency cancer predisposition genes.
(2, 8) and a relevant risk factor that is able to facilitate progression at one or several stages in tumour development, regardless that the virus may not be the sole cause of cancer. Several future prospects including the improved detection and characterization of relevant viral genes have been addressed and identification of significant differences among malignant, benign or normal surrounding tissue should be established (2). Also a major drawback when JCV is analysed is the sensitivity and specificity of the used assays. Therefore, the use of novel technologies, such as polymerase chain reaction (PCR) for detection in real time, would allow further characterization of the process of virus-induced cancerogenesis.

This study aimed to investigate a group of patients with primary brain tumours, colorectal cancers (CRC) and precancerous malignancies. Real time PCR assays were used to detected two particular JCV genomic sequences that might be related to cancer pathogenesis: LT and NCCR. Results from the study supported a non-productive course of the infection with JCV and a positive association of the virus with human neoplasia.

Project approval from the Institutional Ethics Committees was given and all specimens were collected after a signed informed consent.

**Materials and Methods**

**Study group**

Patients who underwent surgical procedures of the central nervous system (n=70), resection of CRC (n=44) or colorectal endoscopy (n=18) were enrolled (**Table 1**). Samples were fresh frozen and paired with matched adjacent mucosa in the case of CRC and colorectal polyps. Adenomatous polyps were also studied (n=5) where present in the CRC patients. Out of the patients with lesions of the central nervous system (CNS), all had primary brain tumours which were: 39 glioblastomas, 19 astrocytomas and 12 oligodendrogliomas (**Table 1**). Tumour type was verified as part of the routine histopathological examination by two independent pathologists.

**Sample collection**

Biopsy samples were placed in a sterile 1.5 ml tubes containing 0.6 ml of transport medium (Cat. No. K-1-1/A, Sacace, Como, Italy) during the surgical procedures and were immediately frozen. Specimens were kept at -20°C for a maximum of 48 hours prior to transportation to the virology laboratory. The collected samples were washed three times with a sterile phosphate buffered saline and were stored at -80°C until an extraction procedure was carried out.

**Extraction procedure**

DNA was obtained from 3-7 mg of tumours tissue subjected to extraction with AquaPure DNA Kit (Cat. No. 732-6340EDU, Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. An internal extraction control (Cat. No. Int-DNA, Primer Design, Southampton, UK) was added to each sample in order to verify the presence of amplifiable DNA during the quantitative real-time assay. In addition at the end of the extraction the quality and quantity of DNA was determined with a spectrophotometer and agarose gel-electrophoresis.

**Detection of LT sequences**

All samples were analyzed for the presence of viral sequences that are part of the gene encoding the LT by SYBR Green real-time polymerase chain reaction (PCR) assay. JCT 1 and JCT 2 primers (12) for detection of JCV were used as a part of a modified system (19). Cycling reactions and fluorescent detection was carried out in an Opticon 2 instrument (MJ Research/Bio-Rad, Hercules, CA, USA). Briefly, the PCR mixture consisted of 25 µl 2X master mix (Platinum SYBR-Green qPCR SuperMix-UDG, Invitrogen, Carlsbad, CA, USA), 20 pmol of each primer, 1 µl ROX (as a reference dye) and approximately 0.5÷1.2 µg of total DNA in a 50 µl total reaction volume. Best signal strength and lowest level of background fluorescence was achieved with the next protocol: 50°C for 2 min, 94°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 30 sec with a plate read at the end of each cycle. A dissociation profile was run after the amplification stage in order to verify the specificity of the products. The samples were gradually heated with an increment of 0.3°C and the change of the fluorescent signal was determined at every step. Brain biopsy DNA from a patient with post mortem histopathological conformation of progressive multifocal leukoencephalopathy was used as a positive control in all PCR tests altogether with JCV genomes, partially sequenced and included in GenBank under accession no. GU222438 and GU145384. Additionally a template free control was included in each run. Specimens were considered positive when a significant increase of the fluorescence above the threshold line was obtained and the determined specific melting temperature was corresponding to the positive control profile with a maximal decrease in the intensity of the signal (-Δl/dT) at 75.7°C.

**Quantification of NCCR sequences**

Samples were further analyzed for the NCCR. A commercially available assay was used (Primer Design, UK) as detection and quantification was performed with the Primer Design TaqMan® based JCV Advanced Kit (Cat. No. Path-JCV, Primer Design, Southampton, UK). Eight quantity control dilutions with control viral genomes from 10^8 down to 10 starting copies per reaction, were used in each run. All samples contained an internal amplification control and results were interpreted according to the manufacturer’s guidelines.

Both qualitative and quantitative PCR experiments were repeated at least twice for verification of the results.

**Results and Discussion**

After extraction DNA concentration was measured and all collected specimens had sufficient amplifiable DNA in the range of 0.2-1.2 µg/µl.

Specific DNA sequences from the LT genomic region of JCV were found and characterized in 9 out of 18 (50.0%)
and in 10 out of 44 (22.7%) patients with polyps and CRC, respectively (Table 1).

In the group of patients with polyps, 5 polyps and 3 adjacent mucosa samples were positive for LT. In only one case the PCR result was positive for both the polyp and the normal tissue (Table 1).

In the CRC group 7 tumour samples, 3 polyp specimens (3 out of 5) and 5 biopsies from the resection lines were found positive for LT (Table 1). In one case all three samples (tumour, polyp, resection line) tested positive; there was a case with positive both the tumour and the polyp; and a case where only the polyp was found positive. In two other patients there were paired positive results (tumour and resection line specimen).

All LT positive samples had a late threshold cycle that varied between 36 and 47, indicative of a very low starting copy number.

None of the tested premalignant lesions or cancer samples was positive for the NCCR. Two adjacent tissues from polyp patients were determined to harbour viral sequences with a very low viral load in the range of 10-1000 starting copies.

The results from the SYBR Green® assay targeted at LT, revealed the highest prevalence of JC viral sequences in the tested 39 glioblastoma patients (48.7%). All positive samples had an increase in the fluorescent signal during the late cycles of the PCR, with a threshold cycle that varied from 36 to 49. The subsequent TaqMan® quantitative assay showed that most of the samples did not yield a NCCR product, although the detection limit of the system was 10 starting copies. Where present, viral copy number was low in the range of 10 to 1000. Notably, only 2 of all NCCR positive specimens were also LT positive ones in the glioblastoma group. Similar results were obtained for the other primary brain tumour groups. Results are presented in details in Table 1.

Reports concerning the presence of JCV sequences in human neoplasia vary widely, as some authors describe a prevalence of more than 80% (11), while others fail to detect viral genomic sequences at all (15) resulting in the absence of a conclusive opinion on the role of JCV for development of human tumours. This research took into consideration several technical details which might explain the observed differences between the research groups. Studies that fail to establish a positive association of JCV with human tumours often isolate DNA from formalin-fixed or paraffin-embedded tumours, where nucleic acids are usually of inferior quality (6). Therefore, the use of fresh or fresh-frozen materials, as we collected, or a laser microdissection technique is recommended (5). Also additives such as Topoisomerase I (10), PCR enhancers, etc. are used in order that the “true picture” is revealed. It has been shown that a low copy number of viral sequences is present in human tumours, necessitating the use of more sensitive assays rather than conventional PCR followed by ethidium bromide staining (5). Authors that identify JCV sequences in experimentally inoculated monkeys that develop brain tumours, also suggest that usually only a single copy of the viral genome is present in each tumour cell in an integrated state (14). Similarly we expected a low number of sequences in our samples and employed assays with high sensitivity such as real-time PCR, giving a better chance for detection of viral genomes. Another novel real-time PCR technique that can be of use due to its high sensitivity and specificity is Light Upon Extension (LUX) real-time PCR (9,18). It should also be considered that studies usually determine a “snapshot” whereas the process of cellular transformation is dynamic and complex.

Initially the samples were tested for sequences that are part of the LT gene. Its product is a key factor for cell cycle disorganization and when genomic sequences that encode for LT are identified this might represent indirect evidence in support of a possible role of JCV in malignant transformation. JCV has been the subject of intense investigation, as it possesses stringent tropism towards the glial cells, the ability to induce “bizarre astrocytes” and formation of brain tumours in PMl patients (16). Thus, the virus is considered a plausible etiologic agent for the development of primary brain tumours (5). Moreover, if replication of the viral genome and formation of mature particles are altered and incomplete, it might be expected that viral sequences would be in low numbers and/or only partial genomic fragments would be present.

The presence of NCCR is essential for the proper replication of JCV. We believe that amplification failure or

**TABLE 1**

<table>
<thead>
<tr>
<th>Number of tested patients</th>
<th>Histology</th>
<th>Gender (M:F)</th>
<th>Mean age in years; range</th>
<th>LT PCR positive samples</th>
<th>NCCR PCR positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>39/24 (61.5%)</td>
<td>Glioblastoma</td>
<td>22:17</td>
<td>53; 2-80</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>19/7 (36.8%)</td>
<td>Astrocytoma</td>
<td>10:9</td>
<td>34; 6-61</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>12/5 (41.7%)</td>
<td>Oligodendroglia</td>
<td>7:5</td>
<td>46; 32-62</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>44/10 (22.7%)</td>
<td>CRC or CRC &amp; polyp</td>
<td>29:15</td>
<td>65; 35-82</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Resection Line</td>
<td></td>
<td></td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>18/9 (50.0%)</td>
<td>Polyp</td>
<td>12:6</td>
<td>62; 33-87</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

M: male; F: female; JCV: JC virus; LT PCR: polymerase chain reaction for detection of partial genomic sequences encoding the large T antigen; NCCR PCR: polymerase chain reaction for detection of partial genomic sequences from the non-coding control region.
low NCCR copy number might confer a prerequisite for incomplete or non-lytic course of the infection. It has been shown that JCV sequences originating from PML patients carry NCCR rearrangements compared to the archetypal strain (14). Similarly, it is possible that changes altering the function of NCCR may occur in brain tumour cells leading to inability for mature virions to be formed. Indeed, it has been shown that under the circumstances of continuous LT gene expression malignant transformation may be induced (4).

The results from this study add some more information as the prevalence of JCV in two groups of Bulgarian patients was determined with a relatively high (50%) prevalence among the polyp patients and a moderate (22.7%) in the group of CRC cases.

A possible role of JCV in the early stages of CRC development is suggested by the study results. There was a higher prevalence among the polyp group compared to the CRC patients, which is in support of such hypothesis. It has previously been reported that the gastrointestinal tract might be an entry site for the virus (7). Moreover, JC viral sequences have been described to be present in the enteric glia (17). Enteric cells normally do not support JCV replication but it can be speculated that JCV might enter such cells. the virus would not replicate but as basal cells are dividing viral genomes might be reproduced in a mechanistic way, altogether with the cellular genome. Results showing the presence of low number of viral genomes in normal mucosa samples are in favour of such speculation. As the synthesis of JCV DNA is not regulated by the cell it is more likely that changes in the viral genome or even integration into cellular genes would appear. Therefore low JCV copy number that diminishes or becomes undetectable as the malignancy develops is expected.

Furthermore, the melting curve analysis depicted the melting temperature profile of the amplified fragments. Five more samples from patients with CRC or polyps that were considered negative, had a pattern that was similar but slightly shifted from the control one. This might be a result from changes in the targeted sequences, but further investigation is needed for clarification.

Conclusions
It remains unclear if JCV has a decisive role in the process of malignant transformation. In our opinion the current results suggest that JCV might induce or play role in stages of malignant transformation. The results support a possible ethio-pathogenetic association of JC virus with colorectal malignancies. A non-productive course of the infection in colorectal cells is suggested as a premise for malignant transformation. Real time PCR technologies possess superior sensitivity when compared to conventional PCR, and studied samples can be quantitatively characterized. This allows better analysis of the experimental results and formation of scientific hypothesis.

Further data in support of the infectious causes of cancer present a new concept that warrants the need for advanced investigations.

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REFERENCES