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Prevalence of JC polyomavirus genomic sequences from the large T-antigen and non-coding control regions among Bulgarian patients with primary brain tumours

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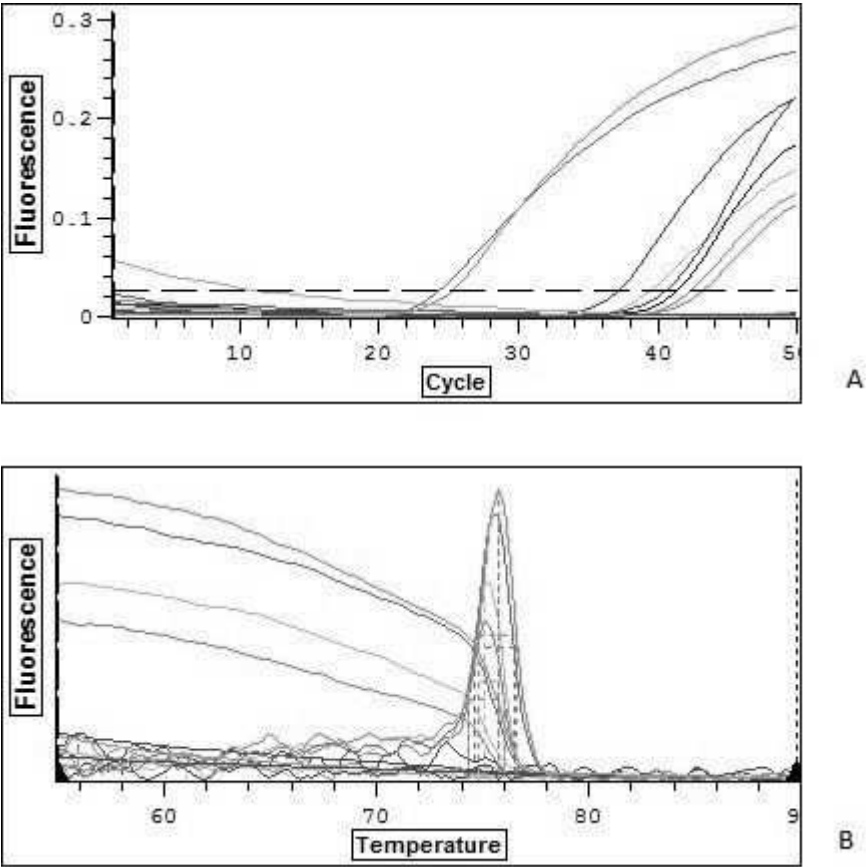


Fig. 1. Real-time SYBR Green polymerase chain reaction assay
A. An amplification plot showing positive control graphs with a threshold at 25th cycle and several positive samples with a threshold in the range from cycle 36 to 44.
B. Melting curve analysis showing a characteristic profile of the positive control at 75.7C identical to the profile of the positive samples

117x118mm (96 x 96 DPI)

Table I

Presentation of the patients according to their histopathological diagnosis, gender, age and prevalence of viral sequences

Tumor/Lesion type	Number	Gender (M:F)	Age (years) mean; range	LT* positive	Mean Ct**; range	NCCR***	
						Positive	Range of copies
Glioblastoma	39	22:17	53 (2-80)	19 (48.7%)	44 36-49	7 (17.5%)	10-100
Astrocytoma	19	10:9	34 (6-61)	6 (31.6%)	41 37-41	3 (15%)	10-100
Oligodendroglioma	12	7:5	46 (32-62)	4 (33.3%)	38 37-40	2 (16.7%)	10-100
Oligoastrocytoma	3	3:0	40 (33-51)	2 (66.7%)	43-44	none	N/A
Medulloblastoma	5	2:3	28 (3-48)	2 (40.0%)	49	none	N/A
Ganglioglioma	5	3:2	24 (11-54)	2 (40.0%)	39-41	3 (60.0%)	10-100
Ependimoma	5	2:3	45 (37-53)	none	N/A	2 (40.0%)	10-100
Pineocytoma	3	2:1	27 (7-59)	1 (33.3%)	40	2 (66.7%)	10-100
Radiotherapy induced	2	2:0	50 (45, 56)	2 (100%)	40	1 (50.0%)	10

alterations							
Metastases	6	4:2	50 (2-65)	1 (16.7%)	48	none	N/A
Rare brain tumors	6	4:2	34 (4-73)	None	N/A	1 (16.7%)	10
Vascular and other malformations	7	4:3	37 (17-62)	1 (14.3%)	43	none	N/A

*LT: Large T antigen encoding genomic sequences detected by SYBR Green real-time polymerase chain reaction;

**Ct: threshold cycle determined during the SYBR Green real-time polymerase chain reaction;

***NCCR: DNA sequences from the non-coding control region determined by quantitative assay;

Table II

Presentation of the patients who were determined as both LT and NCCR positive

Tumor type	Sex and age	Ct	Number of NCCR copies
Glioblastoma	57/F	36	10-100
Glioblastoma	67/M	50	10
Radiotherapy induced alterations in a patient with Glioblastoma	45/M	39	10
Astrocytoma	51/F	37	10-100
Astrocytoma	58/M	41	10
Oligodendroglioma	41/F	38	10-100
Ganglioglioma	25/F	41	10-100
Pineocytoma	15/F	40	10-100

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Prevalence of JC polyomavirus genomic sequences from the large T-antigen and non-coding control regions among Bulgarian patients with primary brain tumours

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Running head: Prevalence of JCV in primary brain tumours

ABSTRACT

A total of 111 fresh brain biopsies from patients with primary brain tumours were examined for JC polyomavirus (JCV) sequences from the Large T antigen encoding region (LT) and the viral non-coding control region (NCCR). SYBR Green and TaqMan real-time polymerase chain reaction assays were used. In the glioblastoma group of 39 patients 48.7% were positive for LT sequences. Among the astrocytoma group (19 patients) and the oligodendroglioma group (12 patients) 31.6% and 33.3% were also positive. The prevalence of LT genomic sequences among the other groups was as follows: in 2 out of 3 oligoastrocytomas; in 3 out 5 gangliogliomas; in 2 out of 5 meduloblastomas; in 1 out 3 pineocytomas; and in none of the tested 5 ependimomas. All positive samples had a late threshold cycle that varied from 36 to 49, indicative of very low starting viral number. Only 21 of all the 111 samples were positive for NCCR. Low copy number in range of 10 to 1000 was present. Notably, only 8 of all NCCR positive specimens were also LT positive. It might be suggested that the disproportion between the results for LT and NCCR is either due to clonally integrated LT fragments, with loss of genetic material, or changes in the NCCR. The latter would alter the productive course of the infection and may establish a premise for continuous interaction of viral regulatory proteins with cell molecules that are responsible for the control of the cell cycle. This may lead subsequently to malignant transformation.

Key Words: brain tumours; JC virus; oncogenesis; progressive multifocal leukoencephalopathy

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INTRODUCTION

Human polyoma viruses (HPoV) are small oncogenic viruses, comprised of an icosahedral capsid around a circular, double-stranded DNA molecule. Their genome is approximately 5100 base pairs in length and can be divided into a non-coding control region (NCCR) and overlapping genes encoding the viral regulatory and structural proteins. There are no genes encoding viral replication enzymes. Therefore, the replicative strategy of HPoV relies on the induction of an S phase environment and stimulation of the cellular machinery for specific enzyme production. This is a complex process involving mainly the so called large T-antigen (LT) [Imperiale and Major, 2007]. This peptide can interact with several important regulatory cell molecules by binding to tumours suppressor proteins like pRb and p53 [Khalili et al., 2006; White and Khalili, 2004] on the one hand; and to proto-oncogene products such as IRS-1 and β -catenin, [Lassak et al., 2002],[Gan and Khalili, 2004] on the other. It is believed that this interference with the cell cycle regulation might be a key factor for induced malignant transformation by viruses.

The primary infection with JC viruses (JCV), from the HPoV family, has been established to occur during childhood. The presence of specific JCV antibodies is pointed out by zur Hausen to be up to 98% of the human population [zur Hausen, 2008]. The infection remains latent but under certain conditions a viral reactivation is possible. The production of mature JC viral particles, followed by the death of the infected cells can lead to progressive multifocal leucoencephalopathy [Imperiale and Major, 2007]. If the infected cells die, any possible disregulation has no consequence for cellular

1 transformation. However, when the viral replication is in some way impaired and
2 associated with the survival of the affected cell, the events causing disturbances in
3 regulation contribute to the process of cellular transformation [Nevins, 2007]. JC virus
4 has a marked oncogenic potential when cultured *in vitro*. The development of tumours
5 in inoculated or transgenic experimental animals has also been demonstrated [Miller et
6 al., 1984].

7 JCV possess oncogenic potential, but still their role concerning the induction of
8 primary brain neoplasia is unclear and findings reported in the literature vary widely.
9 Investigations on the effect of JCV cultured *in vitro* and on laboratory animals rise
10 evidence associating this virus with human tumours and offer possible mechanisms of
11 JCV-mediated cell transformation [Del Valle et al., 2008]. In contrast, some researchers
12 fail to identify genomic sequences in human tumours [Herbarth et al., 1998; Rollison et
13 al., 2005]. Although a possible “hit-and-run” mechanism is discussed, they put under
14 question an association of these viruses with malignant transformation. There are
15 obstacles that hamper the establishment of a causal role between JCV and human
16 neoplasia. For instance, the discrepancy between the high prevalence of HPoV in nature
17 and the relatively low frequency of JCV-associated cancer has been raised as an
18 argument against a direct association between the two phenomena. Most often the
19 primary JCV infection is subclinical, making it difficult to establish the exact moment
20 and mechanism of infection. The incubation period between the onset of the infection
21 and the appearance of the tumours might be extended to several decades. In addition, a
22 productive JCV infection cannot be reproduced in laboratory animals, limiting
23 investigations aimed to give answers to the above questions. Environmental (e.g., co-
24 carcinogens) or host factors (e.g., immune system status) can additionally modify the

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viral pathogenesis, making it even more difficult for experimental research [Khalili et al., 2006].

Criteria about the etiologic role of an infectious agent and a neoplastic disease have been proposed: [zur Hausen, 2006]

1. Epidemiological plausibility and evidence that the viral infection is a risk factor for the development of a certain tumours type;
2. Regular identification and persistence in the cells of a particular tumours of the respective infectious agent's nucleic acids;
3. Cell proliferation stimulation following transfection with given genomes or sequences in the corresponding tissues and cell types;
4. Demonstration that the proliferative induction and the malignant phenotype of a certain tumours cells depend on functions executed by the persisting nucleic acids of the respective infectious agent.

There is evidence that JCV meets at least the last three criteria presented [Khalili et al., 2006].

Differences in the patient populations and the used laboratory techniques have been suggested as possible explanations for inconsistencies in the results across different researchers [Del Valle et al., 2008; Rollison et al., 2005]. It has been generally considered that additional facts are needed as data about the presence of polyoma virus JC in primary brain tumours samples differ among the studied groups worldwide.

The aim of this study was to determine the prevalence of JC polyomavirus sequences among patients with primary brain tumours and to evaluate the positive samples in terms of viral copy number.

Project approval from the Ethics Committee of Medical University of Sofia was given and all specimens were collected after a signed informed consent.

MATERIALS AND METHODS

Study group

A total of 111 fresh brain biopsy materials were collected from patients who underwent surgical procedures of the central nervous system.

Ninety patients had primary brain tumours, six were diagnosed with a brain metastasis, 6 were with other rare tumours localized in the brain and seven patients had vascular and other malformations. Brain tumour type was verified as part of the routine histopathological examination in St. Ivan Rilski University Hospital by two independent neuropathologists. The study group is presented in details in Table I.

Sample collection

During the surgical procedure 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) 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

1 presence of amplifiable DNA during the quantitative real-time assay. In addition at the
2 end of the extraction the quality and quantity of DNA was determined with a
3 spectrophotometer and agarose gel-electrophoresis.

5 **Detection of LT sequences**

6 All samples were analyzed for the presence of viral sequences that are part of the gene
7 encoding the LT by SYBR Green real-time polymerase chain reaction (PCR) assay. JCT
8 1 and JCT 2 primers [MacKenzie et al., 2003] for detection of JCV were used as a part
9 of a modified system. Cycling reactions and fluorescent detection was carried out in an
10 Opticon 2 instrument (MJ Research/Bio-Rad, Hercules, CA, USA). Briefly, the PCR
11 mixture consisted of 25 µl 2X master mix (Platinum SYBR-Green qPCR SuperMix-
12 UDG, Invitrogene, Carlsbad, CA, USA), 20 pmol of each primer, 1 µl ROX (as a
13 reference dye) and approximately 0.5÷1.2 µg of total DNA in a 50 µl total reaction
14 volume. Best signal strength and lowest level of background fluorescence was achieved
15 with the next protocol: 50⁰C for 2 min, 94⁰C for 10 min, followed by 50 cycles of
16 denaturation at 95⁰C for 15 sec and annealing/extension at 60⁰C for 30 sec with a plate
17 read at the end of each cycle (Fig. 1A). A dissociation profile was run after the
18 amplification stage in order to verify the specificity of the products. The samples were
19 gradually heated with an increment of 0.3⁰C and the change of the fluorescent signal
20 was determined at every step. Both negative (DNA free) and positive (JCV genomes,
21 partially sequenced and included in GenBank under accession no. GU222438 and
22 GU145384) samples were used in each run. Specimens were considered positive when a
23 significant increase of the fluorescence above the threshold line was obtained and the
24 determined specific melting temperature was corresponding to the positive control

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1 profile with a maximal decrease in the intensity of the signal (-dI/dT) at 75.7⁰C (Fig.
2 1A, Fig. 1B).

3
4 **Quantitation of NCCR sequences**

5 Samples were analyzed further for the NCCR. Detection and quantitation was
6 performed with the Primer Design TaqMan[®] based JCV Advanced Kit (Cat. No. Path-
7 JCV, Primer Design, Southampton, UK). A total number of 8 quantity control dilutions
8 with the respective control viral genomes, covering a spectrum of 10⁸ down to 10
9 starting copies per reaction, were used in each run. All samples contained an internal
10 amplification control and results were interpreted according to the manufacturer's
11 guidelines.

12
13 Both qualitative and quantitative PCR experiments were repeated at least twice
14 for verification of the results.

RESULTS

All collected specimens were determined to contain sufficient amplifiable DNA in the range of 0.2-1.2 µg/µl.

The results from the SYBR Green[®] assay targeted at LT, revealed the presence of JC viral sequences in a total of 41 out of the 111 tested samples. The distribution of LT positive patients among the different groups varied in a broad range. Notably, 48.7% (n=19) from the tested 39 glioblastoma patients were positive for LT sequences. Another six in the astrocytoma group of 19 subjects, and four positive samples in the oligodendroglioma group of 12 were determined. These formed a prevalence of 31.6% and 33.3%, respectively. Two out of three samples from patients with oligoastrocytoma were also positive for LT, as well as two out of five in the groups of medulloblastoma and ganglioglioma patients. None of the tested five ependimomas and only one from the three pineocytoma harboured LT genomic sequences. Two patients, who underwent radiotherapy following neurosurgery for an astrocytoma and glioblastoma are presented separately. The result from the LT PCR was positive for both cases. The prevalence of JCV sequences among the study groups is summarised in details on Table I. Notably, 48.7% from the tested 39 glioblastoma patients were positive for LT sequences. The prevalence of JCV sequences among the study groups is presented in details on Table I. Two patients, who underwent radiotherapy following neurosurgery for an astrocytoma and glioblastoma are presented separately. The result from the LT PCR was positive for both cases.

In comparison to the primary brain tumours several (n=6) rare tumours with brain localisation (primary plexus choroideus carcinoma, central neurocytoma, estesioneuroblastoma, germinoma, teratoma with malignant transformation and

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1 retinoblastoma) were also tested and none of them was found to yield a PCR
2 amplification for LT. Out of these tumours only in the retinoblastoma patients NCCR
3 was amplified. Six other tumours were diagnosed to be CNS metastases with different
4 place of origin. Only one sample (metastasis from a papillary carcinoma) was evaluated
5 as LT positive. JCV sequences were also found in a brain biopsy from a patient who
6 underwent surgery because of an arterio-venous malformation (Table I).

7 All positive samples had an increase in the fluorescent signal during the late
8 cycles of the PCR, with a threshold cycle that varied from 36 to 50 (Table I).

9 The subsequent TaqMan[®] quantitative assay showed that most of the samples
10 did not yield a NCCR product, although the detection limit of the system was 10
11 starting copies. Only 21 of all the 111 samples were documented as NCCR positive
12 (Table I). Low copy number in the range of 10 to 1000 was present. Notably, only eight
13 of all NCCR positive specimens were also LT positive (Table II).

DISCUSSION

Reports concerning the presence of HPoV sequences in human neoplasia vary widely resulting in the absence of a conclusive opinion on the role of these viruses for development of brain 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 [Ferrer et al., 2007]. Therefore, the use of fresh or fresh-frozen materials or a laser microdissection technique is recommended [Del Valle et al., 2008]. 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 [Del Valle et al., 2008]. Authors that identify JCV sequences in monkeys inoculated experimentally 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 [Miller et al., 1984]. Similarly we expected a low number of sequences in the tested 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 [Kalvatchev et al., 2010].

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 possess stringent tropism towards the glial cells, the ability to induce

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1 “bizarre astrocytes” and formation of brain tumours in progressive multifocal
2 leukoencephalopathy patients [Richardson, 1961]. Thus, the virus is considered a
3 plausible etiologic agent for the development of primary brain tumours [Del Valle et al.,
4 2008]. If replication of the viral genome and formation of mature particles are altered
5 and incomplete, it might be expected that viral sequences would be in low numbers
6 and/or only partial genomic fragments would be present.

7 The presence of NCCR is essential for the proper replication of HPoV.
8 Amplification failure or low NCCR copy number might confer a prerequisite for
9 incomplete or non-lytic course of the infection. It has been shown that JCV sequences
10 originating from progressive multifocal leukoencephalopathy patients carry NCCR
11 rearrangements compared to the archetypal strain [Miller et al., 1984]. Similarly, it is
12 possible that in brain tumour cells changes altering the function of NCCR may occur
13 leading to inability for mature virions to be formed. Indeed, it has been shown that
14 under the circumstances of continuous LT gene expression malignant transformation
15 may occur [Del Valle et al., 2001a]. A similar mechanism of viral-induced oncogenesis
16 was proposed for Kaposi's sarcoma associated herpesvirus [Zhao et al., 2007]. The
17 authors described that the disrupted control of lytic replication genes led to a non-lytic
18 course of the infection and a subsequent tumorigenesis. However, exact molecular
19 interactions and its significance *in vivo* are so far not well understood.

20 The histopathological diagnosis revealed one of the studied tumours to be
21 subependymal giant cell astrocytoma. This is a rare type of brain tumour that commonly
22 occurs in the setting of the familial syndrome of tuberous sclerosis complex and
23 hereditary factors are considered to explain its development [Adriaensen et al., 2009].
24 Therefore, as expected viral sequences were not detected. Similarly, in other rare brain

1 tumours JCV sequences were also not found, as probably human genetic factors might
2 have a prevalent impact for the development of malignant transformation.

3 Samples were often processed and tested prior the definitive histological
4 diagnosis, so six tumours, were later determined to be actually metastases. It might be
5 expected that these would be JCV negative, as it was the case with five of them.
6 However, one sample was positive for LT. The logic behind this remains unclear so far.

7 Two of the patients had alterations that were induced by the radiotherapy
8 following the surgical resection for fibrilar astrocytoma and glioblastoma. Their
9 biopsies were determined to be LT positive. Also in a non tumour disease, where
10 surgery was performed due to arterio-venous malformation JCV LT fragments were
11 found. These results bring into concern a question regarding the role of the immune
12 system in controlling viral re-activation. It has been suggested that JCV is already
13 present in a latent state in the brain tissue [Lam et al.]. Most probably under conditions
14 of impaired cellular immunity viral reactivation may occur. If a severe immune
15 deficiency is present progressive multifocal leukoencephalopathy might develop.
16 However, it can be speculated that the immune response can help restrain the viral
17 reproduction and bring the viral infection again to a latent state. If it is assumed that
18 several reactivation-restriction cycles might be repeated there is a risk that viral
19 genomic element would be altered. Consequentially, uncontrolled expression of
20 regulatory elements such as LT might occur. If virions, which would normally trigger
21 the immune system, do not appear, this could result in a premise for malignant
22 transformation of the infected cell. This hypothesis is also supported by a recent
23 investigation of polyomaviruses-induced tumours in mice, where NK cells and gammadelta
24 T cells mediated resistance to formation of the tumours [Mishra et al.]. Although this

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1 investigation did not perform an expression assay in order to show the activity of LT,
2 other researchers have also demonstrated that LT is expressed in brain tumour tissue
3 [Del Valle et al., 2001b].

4 It remains unclear if JCV has a decisive role in the process of brain cell
5 malignant transformation. The current results suggest that JCV might induce or play
6 role in the subsequent stages of malignant transformation.
7 Further data in support of the infectious causes of cancer present a new concept that
8 warrants the need for advanced investigations.

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