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Index

Appendices

APPENDIX A

LIST OF PUBLICATIONS

1. **Chattaraj, S.** and Bhattacharjee, S. 2014. Molecular analysis of JC Polyomavirus genotypes circulating among tribal populations of North-Eastern West Bengal, India. *Polish Journal of Microbiology* 63(2): 191-201.

2. **Chattaraj, S.**, Bera, N. K., Dutta, C. and Bhattacharjee, S. 2015. Quantification of human polyomavirus JC virus load in urine and blood samples of healthy tribal populations of North-Eastern part of West Bengal, India. *Indian Journal of Medical Microbiology* 33(4): 491-495.

3. Bhattacharjee, S and **Chattaraj, S**. 2017. Entry, infection, replication, and egress of human polyomaviruses: an update. *Canadian Journal of Microbiology* 63: 1-19.

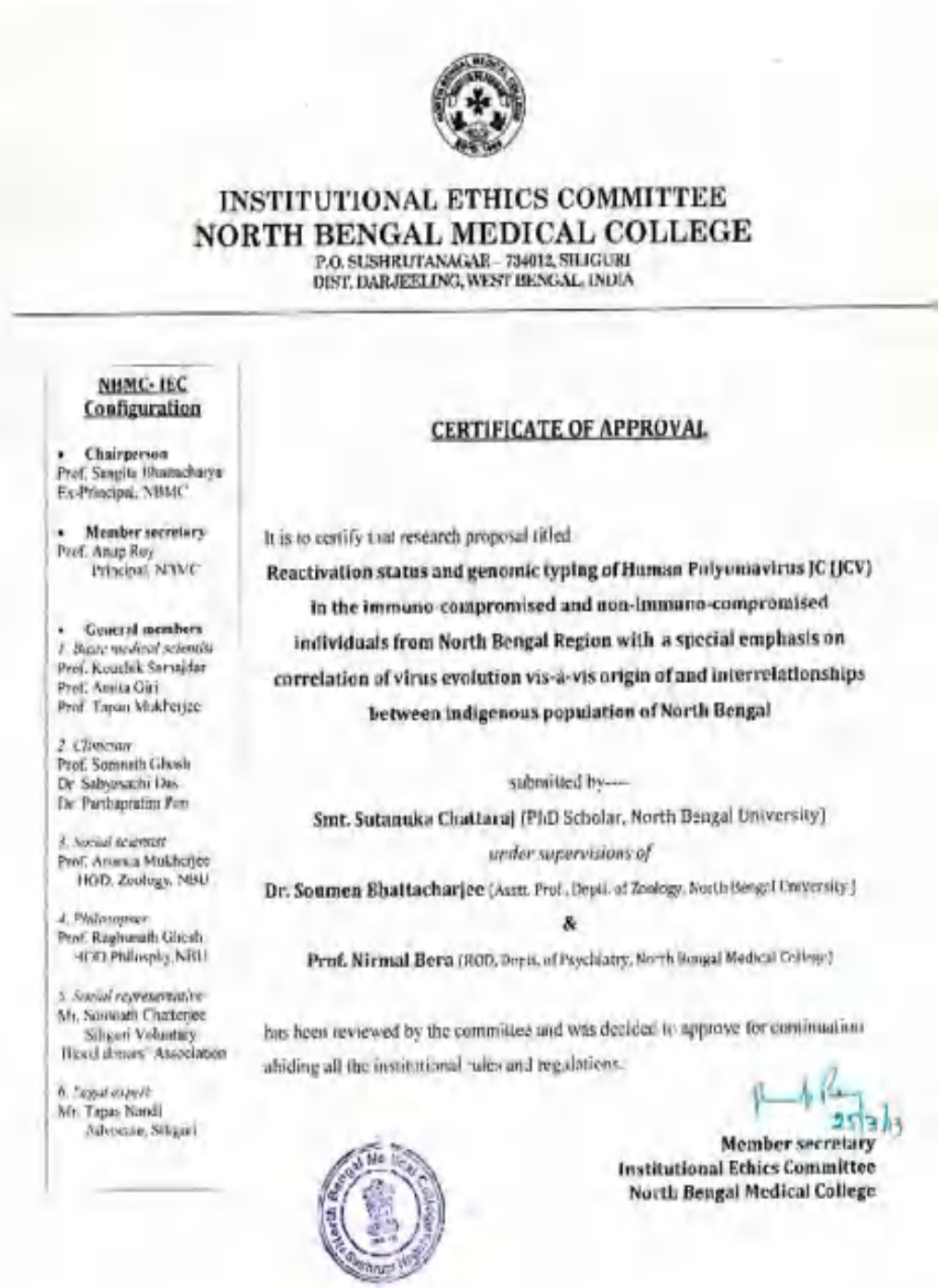
4. Paul, S., Modak, D., **Chattaraj, S**., Nandi, D., Sarkar, A., Roy, J., Chaudhuri, T. K. and Bhattacharjee, S. 2021. Aloe vera gel homogenate shows anti-inflammatory activity through lysosomal membrane stabilization and downregulation of TNF-α and Cox-2 gene expressions in inflammatory arthritic animals. *Future Journal of Pharmaceutical Sciences* 7: 12-19.

APPENDIX B

(SEMINARS AND CONFERENCES)

1. **Chattaraj, S.** and Bhattacharjee, S. Analysis of Non-coding control region of prevalent JC Polyomavirus in North-Eastern part of West Bengal, India. UGC sponsored seminar on "Biological Research in Human Welfare" organized by Department of Zoology, university of North Bengal, Siliguri-734013, District-Darjeeling, West Bengal on February 7, 2014. [Poster Presentation].
2. **Chattaraj, S.** and Bhattacharjee, S. Molecular Epidemiological survey of Human Polyomavirus JC (JCV) in the healthy tribal populations of North Eastern West Bengal, India. International Symposium on "Genetic Analysis Translational and Developmental" and Annual Meeting of Society for Biotechnolgists (India) organized by Department of Zoology, University of Burdwan, Burdwan-713104, West Bengal on November 21-23, 2014. [Poster Presentation].
3. **Chattaraj, S.** Bera, N. and Bhattacharjee, S. Quantification of Human Polyomavirus JC (JCV) DNA load in healthy tribal populations of Nort-Eastern West Bengal, India. National Conference on "Applied Zoology in Sustainable and Development: an update" organized by Department of Zoology, university of North Bengal, Siliguri-734013, District-Darjeeling, West Bengal on January 30 – February 02, 2015. [Oral Presentation].

APPENDIX C



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CERTIFICATE OF APPROVAL

It is to certify that research proposal titled

Reactivation status and genomic typing of Human Polyomavirus JC (JCV) in the immuno-compromised and non-immuno-compromised individuals from North Bengal Region with a special emphasis on correlation of virus evolution vis-à-vis origin of and interrelationships between indigenous population of North Bengal

submitted by—

Smt. Sutanuka Chattaraj (PhD Scholar, North Bengal University)

under supervisions of

Dr. Soumen Bhattacharjee (Asstt. Prof., Deptt. of Zoology, North Bengal University)

&

Prof. Nirmal Bera (HOD, Deptt. of Psychiatry, North Bengal Medical College)

has been reviewed by the committee and was decided to approve for continuation abiding all the institutional rules and regulations.

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West Bengal, India, PIN – 734 013

Human Ethical Committee Certificate

The research proposal submitted by **Dr. Soumen Bhattacharjee (PI)** and Dr. Tushar Chakraborty (Co-I) entitled *"Reactivation status and genomic typing of Human Polyomavirus JC (JCV) in the immunocompromised and non-immunocompromised individuals from North Bengal region with a special emphasis on correlation of virus evolution vis-à-vis origin of and interrelationships between indigenous populations of North Bengal"* was considered and discussed by the members of the **Human Ethical Committee** in its meeting held on 18.11.2009 under **Agenda** 1, at the Department of Zoology, University of North Bengal, Siliguri, West Bengal. The members of the committee approved the research project proposal subject to time to time monitoring.

11.12.2009

(Prof. J. Pal)
Head
Zoology Department
N.B.U.
(Convener)

Head
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APPENDIX D

(21)

CONSENT FORM II

PARTICIPANT CONSENT FORM

Participant's name: Roshadi kiran Address: Mayha Tea Estate

Title of the project:

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the participant: _____ Date: 18/11/11

Signature of the witness: _____ Date: 18/11/11

(Note: Consent form II should be appropriately worded for adults and children (less than 18 years), e.g. If the participant is less than 18 years of age, instead of 'my participation', 'my child's/ward's participation' needs to be explicated.)

CONSENT FORM II

PARTICIPANT CONSENT FORM

Participant's name: Address:

Title of the Project: "Reactivation status and Genomic Typing of ... North Bengal" [CSIR Sanction Letter No. 37(1445)/10/EMR-II dated 09.12.2010]

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the participant: Sayanti Narjinary Date: 16/6/2012

Signature of the witness: Date:

(Note: Consent form II should be appropriately worked for adults and children (less than 18 years) e.g. if the participant is less than 18 years of age, instead of 'my participation', 'my child's/ward's participation' needs to be replaced.)

CONSENT FORM II

PARTICIPANT CONSENT FORM

Participant's name: Address:

Title of the Project: "Reactivation status and Genomic Typing of ... North Bengal" [CSIR Sanction Letter No. 37(1445)/10/EMR-II dated 09.12.2010]

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the participant: Poulomi Mukherjee Date: 16/6/12

Signature of the witness: Date:

(Note: Consent form II should be appropriately worked for adults and children (less than 18 years) e.g. if the participant is less than 18 years of age, instead of 'my participation', 'my child's/ward's participation' needs to be replaced.)

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ORIGINAL PAPER

Molecular Analysis of JC Polyomavirus Genotypes Circulating Among Tribal Populations of North-Eastern West Bengal, India

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Abstract

There is a resurgence of interest in the study of occurrence, genotype and pathogenic associations of human Polyomaviruses in recent years. In the present study, we have ascertained the presence of human Polyomavirus JC (JCV) in the urine and peripheral blood leukocytes of tribal populations, for the first time in the North-Eastern part of West Bengal State of India. We have also characterized the prevalent genotypes of the non-coding control regions (NCCRs) of these natural isolates. The result suggests a high incidence of JCV reactivation in the populations assayed. Approximately 25% of the non-immunocompromized tribal men and women, tested positive based on polymerase chain reaction (PCR) analysis, and these results were further confirmed by sequencing of PCR products. Pairwise sequence comparison and alignment of the NCCR sequence of these Indian strains appeared to be comparable and related to the archetypal JCV (CY) and the Tibetan LH3 strains, with some alterations in few key positions. The sequence analyses were done with regard to transcription factor binding to DNA sequence elements of endemic JCV NCCRs.

Key words: JC Polyomavirus, NCCR, North-East India, TFBS

Introduction

JC virus (JCV), a member of the *Polyomaviridae* family, was first isolated from brain tissue of a 38 year old man with initials – JC, who was suffering from Hodgkin's disease for a quite a long time (Padgett *et al*., 1971). JC virus has simple genome containing a single molecule of covalently closed, circular double-stranded DNA of about 5000 base pair in length (Frisque *et al.*, 1984). The viral genome comprises early and late coding regions that are controlled by a common Non-Coding Control Region (NCCR), which lies between them. The early proximal side of the NCCR is highly conserved and contains the origin of viral DNA replication. The late proximal side of the NCCR contains the repetitive enhancer elements and undergoes rearrangements that account for most of the differences between different strains of the same virus.

Transcriptional regulation of the JCV early and late promoters during the viral lytic cycle is a complicated event that requires participation of both viral key proteins and cellular transcription factors. Several transcription factors are implicated in the regulation of JCV gene expression which include NF-κB (Ranganathan and Khalili, 1993), NFAT4 (Wollebo *et al*., 2012), upstream Target or up-TAR (Chowdhury *et al*., 1993), Tst-1 (Wegner *et al*., 1993), Sp-1 (Henson *et al*., 1992), Spi-B (Marshall *et al*., 2010), GBP-i (Raj and Khalili, 1994), Y-box binding protein 1 (YB-1) and Purα (Chen and Khalili, 1995), Nuclear factor 1 or NF-1 (Amemiya *et al.* 1989), CREB/ATF-1 (Lonze and Guinty, 2002), Activator Protein 1 (AP-1) family members (Sadowska *et al*., 2003), p53 (Ariza *et al*., 1994), Early growth response-1 protein or Egr-1 (Romagnoli *et al*., 2008), Bcl-2-associated athano gene-1 or BAG-1 (Devireddy *et al*., 2000) and CAAT/enhancer binding protein beta or C/EBPβ (Romagnoli *et al*., 2009).

JC polyomavirus is widespread in the human population and causes a rare fatal brain infection known as Progressive Multifocal Leucoencephalopathy (PML). PML occurs mainly in a limited number of individuals with suppressed immune system, especially in those with Human Immunodeficiency Virus (HIV) infection/ AIDS (Hou and Major, 2000; Khalili *et al*., 2006) and involves productive infection in both oligodendrocytes and astrocytes. However, there are reports where

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researchers have shown that JCV can even induce PML in non-immunocompromised individuals without AIDS, such as patients receiving monoclonal antibody natalizumab (Langer-Gould *et al*., 2009). JCV exists in ten or more geographically based genotypes identified in the United States, Africa, Europe and Asia (Agostini *et al*., 1997; Sugimoto *et al*., 2002). There appear to be several Asian subtypes and the Indian subtype has been designated as Type 2D (Cui *et al*., 2004).

In this study, we have screened urine and blood samples from tribal/ethnic human population of north-eastern part of West Bengal state of India to record for the first time the JCV reactivation status and to ascertain the prevalent nature of endemic viral NCCRs. Specific oligonucleotide primers were used to amplify the JCV NCCRs by PCR and then sequenced to analyze their genetic architectures. We have recorded about 25% of the subjects to be positive for JCV NCCRs. Analyses of predicted transcription factor binding sites (TFBS) revealed additional putative promoter elements in endemic NCCRs capable of binding to a diverse set of human/vertebrate transcription factors. Further *in vivo* and *in vitro* experiments are expected to substantiate these findings.

Experimental

Materials and Methods

Sample collection. The study methodology was approved by our institutional Human Ethical Committee. A total of 77 samples were collected from the tribal, non-immunosuppressed populations of the north-eastern part of West Bengal State of India with their prior informed consent. The samples included either urine or blood specimens from each individual. The collected samples were from two tribal groups of North Bengal – Oraon and Mundas of Kiran Chandra tea garden, Naxalbari, Darjeeling district (26°41'N Lat; 88°16'E Long) and Rabha or Rava population of Poro Busty, Tufanganj, Coochbehar district (26°18'N Lat; 89°39'E Long). The urine samples were collected in 1.5 ml eppendorf tubes and about 1 ml of blood samples were collected in vials containing EDTA. All the samples were brought to the laboratory and stored at –20°C refrigerator prior to DNA isolation.

DNA Isolation. DNA isolation from urine and blood samples was done using the High Pure Viral Nucleic Acid Kit (Roche Diagnostic GmbH, Germany) as per the manufacturer's instruction. In brief, 200 µl of freshly prepared working solution (carrier RNA-supplemented binding buffer) and 50 µl of Proteinase K solution was added to 200 µl of sample and incubated at 72°C for 10 minutes. Then 100 µl of binding buffer was added and centrifuged at 8,000 × g for 1min. The flowthrough liquid was discarded and 500 µl of inhibitor removal buffer was added and centrifuged again at 8,000 × g for 1 min. After discarding the flow through, samples were washed twice with 450 µl wash buffer. The last step included addition of 50 µl elution buffer followed by centrifugation at 8,000 × g for 1 min. The isolated DNA samples were stored in –20°C refrigerator until used for PCR amplification.

Standard Viral DNA. Plasmid pMITC-BSMKS containing the whole JCV genome (JCV Mad-1 strain) was a gift from Richard J. Frisque, Department of Biochemistry, Microbiology, Molecular & Cell Biology, Pennsylvania State University, USA. The plasmid pGMTW3JCV containing the whole genome of JCV (JCV Taiwan-3 strain) was gifted by Deching Chang, National Chung Cheng University, Taiwan.

PCR amplification and Electrophoretic analysis of endemic JCV NCCRs. Two oligonucleotide primers namely SDJ1 (5'-CCCTATTCAGCACTTTGTCC-3') and SDJ2 (5'-CAAACCACTGTGTCTCTGTC-3') were used for the amplification of the viral control region DNA. A final volume 50 µl PCR reaction mixture contained 20 pM of each primer, 200 µM dNTPs (NEB, USA), 10X standard Taq buffer containing $MgCl_2$ (NEB, USA) and Taq Polymerase enzyme (NEB, USA). Twenty microlitres of isolated DNA was used for each PCR amplification reaction. Amplifications were performed in an Applied Biosystems Thermal Cycler (Model: 2720 Thermal Cycler, Life Technologies, USA) programmed for initial heating at 94°C for 5 min, 25 cycles at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. The cloned JCV genome plasmids pMITC-BSMKS or pGMTW3JCV were used as positive control in each reaction and sterile distilled water was used as negative control. PCR products were run on 1% agarose gel containing 0.5 µg/ml of Ethidium bromide at 80 V for 1 hour approximately in a Bench Top Lab Systems (Model: BT-MS-300, Taiwan) electrophoresis apparatus and then visualized in a UV transilluminator (Spectroline BI-O-Vision UV/White Light Transilluminator, NY, USA).

Sequence Analysis. Sequencing of the JC virus NCCR PCR product from each positive sample was done at least twice by dye-dideoxy automated chain termination method (Biolinkk, New Delhi, India) and only six of them were deposited in NCBI public domain for GenBank accession. All the remaining NCCR sequences were similar to the ones deposited for GenBank accession. Submission IDs are as follows: NB1 (JX294575), NB2 (JX534216), NB3 (JX534217), NB4 (JX534218), NB5 (JX534219) and NB6 (JX534220). Nucleotide sequences of the endemic JCV NCCRs were aligned with the known NCCR sequences of JCV

strains CY, LH3, Tai3, IN-8 and Mad-1 using DiAlign alignment program of Genomatix suite v2.5 GmbH (Cartharius *et al*., 2005) to compare pairwise similarities (relative to the maximum similarity) among these sequences. All NCCR sequences (T-antigen coding start site to Agnogene coding start site) were again aligned in ClustalX ver. 2.0.3 (Thompson *et al*., 1997) using default parameters, curated in BioEdit ver. 7.0.9.0 (Hall, 1999) to generate an alignment showing deletion/mutation and common transcription factor binding sites derived from experimental data of other research groups. The NCCR sequences of NB3, 4 and 5 being identical only NB3 was included in the multiple sequence alignment. The MatInspector program of Genomatix Software suite v2.5 GmbH (Cartharius *et al*., 2005) was used to search for the predicted transcription factor binding sites (TFBS) in the aligned portion of endemic using a large library of weight matrices based on general core promoter and vertebrate promoter matrix families.

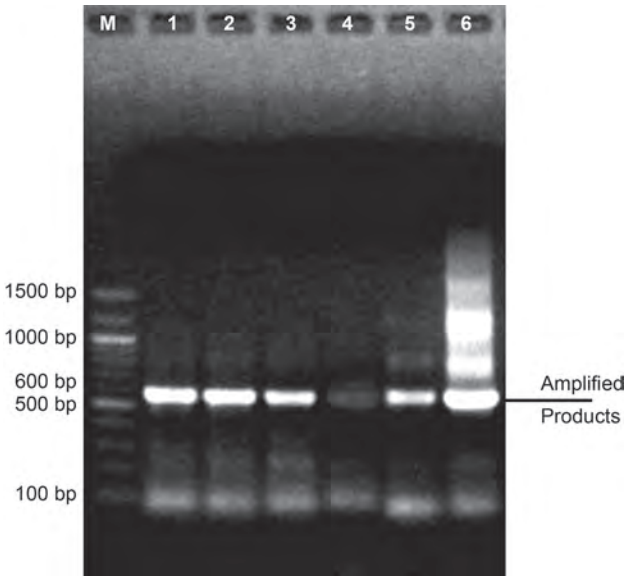

Fig. 1. Agarose gel showing amplified JCV Non Coding Control Region in tribal populations of North-East India.

Lane 1: 100 bp ladder; Lane 2: JCV NB1 NCCR; Lane 3: JCV NB2 NCCR; Lane 4: JCV NB3 NCCR; Lane 5: JCV NB4 NCCR; Lane 6: JCV NB5 NCCR; Lane 7: JCV NB6 NCCR.

Results

PCR amplification and Electrophoretic analyses. PCR amplification of JCV specific NCCR from all urine as well as from blood samples was done to check the prevalence of the virus in the North Bengal region of India. Seventy-seven samples of urine and blood were collected from the tribal, non immunosuppressed populations of this region. Twenty-four samples were from Kiran Chandra tea garden, Naxalbari, Darjeeling district and 53 samples were collected from Poro busty, Tufangunj, Coochbehar district of West Bengal. The two primers SDJ1 and SDJ2 specific for NCCR region amplified a product of approximately 586 bp of DNA. After PCR amplification, products were analysed by agarose gel electrophoresis (Fig. 1). Seven out of 24 assayable samples from the Oraon and Munda tribal groups in Kiran Chandra tea garden were found to be JCV positive showing an overall incidence of 29.17%. In Poro busty, Tufangunj, having mostly Rabha tribal population, 12 out of 53 samples supported positive amplification of the JCV NCCR region having an overall incidence of 22.64% (Table I). Thus the overall incidence of JCV control region DNA in urine and blood of non-immunosuppressed general tribal population was found to be 24.67% (19 out of 77 individuals) in this north-eastern part of India.

Pairwise Comparison of endemic JCV NCCRs with strains reported from North-East and South-East Asia. NCCR region is the variable segment of the viral genome except the early proximal part of the sequence which is highly conserved. In this study, DiAlign program of the Genomatix software suite (Table II) was used to check only the similarity of NCCR sequences among the six endemic isolates of JCV namely NB1, NB2, NB3, NB4, NB5 (from Oraon and Munda Tribes of Kiranchandra Tea Garden, Darjeeling District) and NB6 (from Rabha Tribes of Poro Busty, Coochbehar District) of this region and also with different strains from other regions such as archetypal CY, LH3, Tai3, IN-8 and

Table I
Summary of results obtained in different tribal groups showing incidences of JCV Non-Coding Control Region DNA detection

Location	Tribal group	Sex	Median age	Positive	Negative	Total
Kiran Chandra Tea Garden, Naxalbari, Darjeeling District [26°41'N Lat; 88 °16'E Long; Elev. 772 ft msl]	Oraon and Munda	F = 20 M = 4	F = 26 M = 42	F = 3 M = 4 7 (29.17%)	17	24
Poro Busty, Tufanganj, Coochbehar District [26°18'N Lat; 89 °39'E Long; Elev. 134 ft msl]	Rabha	F = 20 M = 33	F = 59 M = 40	F = 01 M = 11 12 (22.64%)	41	53
Total		F = 40 M = 37	F = 40 M = 26.5	19 (24.67%)	58	77

Table II
Pairwise similarities (relative to the maximum similarity) based on Non-Coding Control Regions (NCCRs) of endemic JCV strains NB1, 2, 3, 4, 5, 6 in comparison to archetype CY, Mad1, LH3, Tai3 and IN8 NCCRs using DiAlign alignment software of Genomatix suite v2.5 GmbH. The number of identical nucleic acids (% of shorter sequence) and maximum values (underlined) are shown

	NB2 JX534216 (372 bp)	NB3 JX534217 (372 bp)	NB4 JX534218 (372 bp)	NB5 JX534219 (372 bp)	NB6 JX534220 (383 bp)	CY AB038249 (384 bp)	LH3 AB262411 (382 bp)	Tai3 U61771 (374 bp)	IN8 AB126992 (384 bp)	Mad1 NC_001699 (393 bp)
NB1|JX294575 (372 bp)	0.943 98%	0.950 98%	0.950 98%	0.950 98%	0.896 94%	0.920 95%	0.927 97%	0.886 94%	0.920 95%	0.721 79%
NB2|JX534216 (372 bp)		0.962 99%	0.962 99%	0.962 99%	0.908 96%	0.921 97%	0.938 98%	0.889 93%	0.915 97%	0.683 80%
NB3|JX534217 (372 bp)			0.976 **100%**	0.976 **100%**	0.909 96%	0.936 97%	0.954 98%	0.904 94%	0.923 97%	0.684 78%
NB4|JX534218 (372 bp)				0.976 **100%**	0.909 96%	0.936 97%	0.954 98%	0.904 94%	0.923 97%	0.684 78%
NB5|JX534219 (372 bp)					0.909 96%	0.936 97%	0.954 98%	0.904 94%	0.923 97%	0.684 78%
NB6|JX534220 (383 bp)						0.962 98%	0.935 95%	0.912 97%	0.954 97%	0.676 78%
CY|AB038249 (384 bp)							0.981 97%	0.965 98%	**1.000** 99%	0.709 79%
LH3|AB262411 (382 bp)								0.961 98%	0.967 97%	0.695 79%
Tai3|U61771 (374 bp)									0.950 98%	0.667 74%
IN8|AB126992 (384 bp)										0.722 79%

* The similarity value 1.000 marks only the two most similar sequences; it does not necessarily mean that these sequences are identical.

Mad-1 strains. It was evident from the study that the NCCR sequences of NB3, 4 and 5 were identical. All the NCCR sequences (NB1-5) except the NB6 paired closely with each other (98–100% similar). Pairwise sequence comparison with other strains revealed that the NCCR sequence of NB1, 2, 3, 4 and 5 were almost similar to the Tibetan LH3 (97–98% similar) NCCR sequence and the NB6 NCCR sequence seemed almost identical with CY strain (98% similarity). These NCCR sequences including that of NB6 were divergent from Tai3, IN-8 and as expected from Mad-1 control regions.

Analyses of NCCRs for Transcription Factor Binding Sites (TFBS). Multiple sequence alignment of endemic JCV NCCRs and other strains reported earlier revealed a 10 nucleotide (169–178) and a di-nucleotide (454–455) deletions in NB1, 2, and 3 (and also in NB5 and NB6) but not in NB6 (Fig. 2). Point mutations in seven different sites within the NCCR of the endemic strains recorded are at 4, 13, 26, 27, 69, 226 and 452 nucleotide positions of the sequence alignment (Fig. 2).

Several transactivating factors have been experimentally shown to bind to JCV NCCR either individually or cooperatively with other cellular factors and/or virus encoded proteins. The binding motifs of NF-κB, NFAT4, upstream Target or up-TAR, Tst-1, Sp-1, Spi-B, GBP-i, Y-box binding protein 1, Purα, Nuclear factor 1 or NF-1, CREB/ATF-1, Activator Protein 1 (AP-1) family members, p53, Early growth response-1 protein or Egr-1, Bcl-2-associated athano gene-1 or BAG-1 and CAAT/enhancer binding protein beta or C/EBPβ are shown on the aligned sequences (Fig. 2). In an effort to check further the binding of putative transcription factors to this control region *in silico* we have searched a well defined general core and vertebrate transcription factor matrix families in MatInspector program of the Genomatix software suite (Cartharius *et al*., 2005). Supplementary Table I shows putative binding sites of transcription factors in an abridged form. Matches for transcription factors that are reported to be active in cells/tissues such as antibody-producing cells, antigen-presenting cells, bone marrow cells, hematopoietic and immune system, leukocytes, lymphocytes, monocytes, myeloid cells, phagocytes, brain, CNS, endocrine system, kidney, nervous system, urinogenital system are retained in the table list.

Discussion

We report here, for the first time, the incidence and sequence characterization of human polyomavirus JCV non-coding control/regulatory regions or NCCRs,

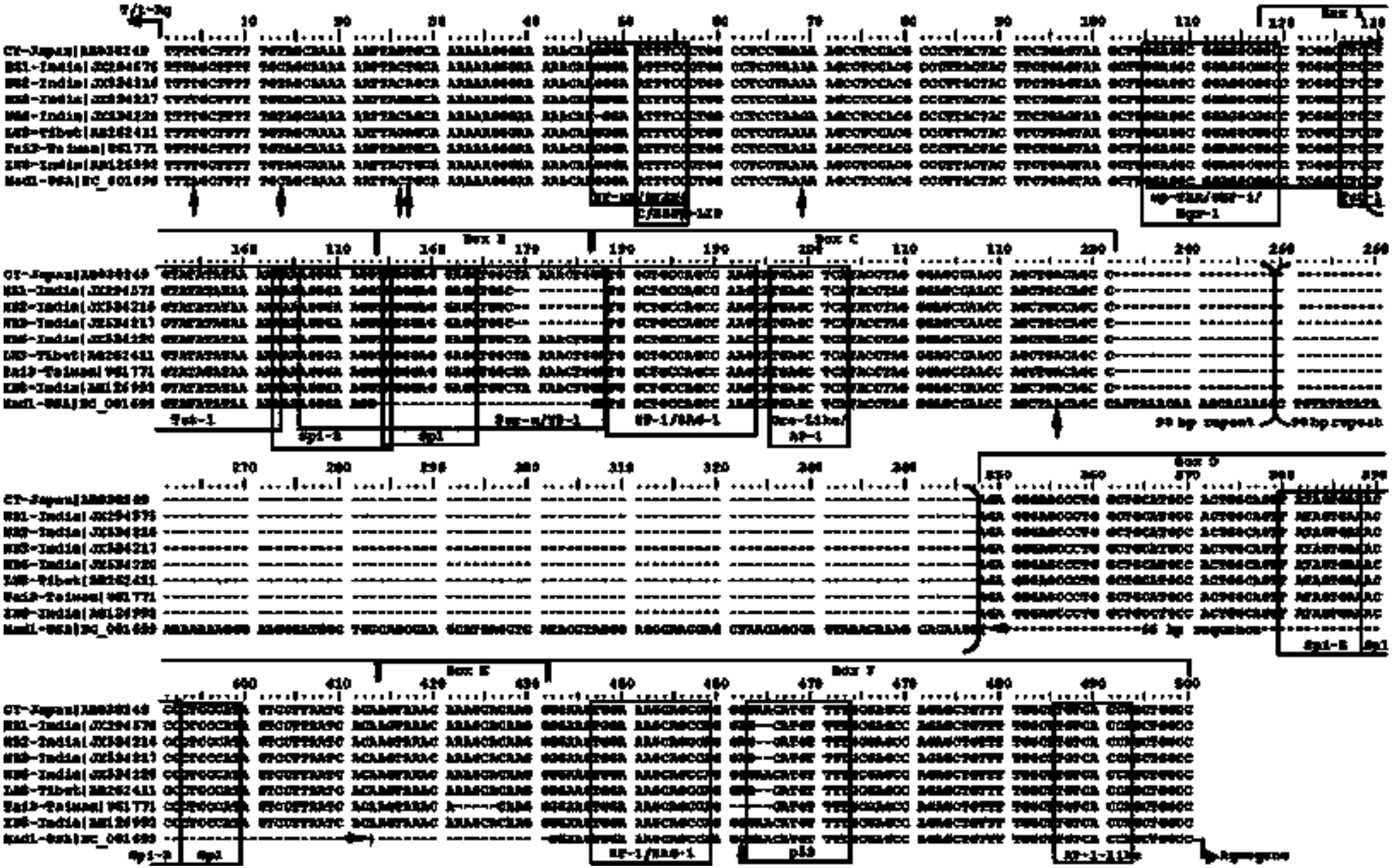

Fig. 2. Non-Coding Control Regions (NCCRs) of JCV endemic strains NB1, NB2, NB3 and NB6 were aligned against the CY, LH3, Tai3, IN-8 and Mad-1 strains, showing the common binding sites of known and reported transcription factors in square blocks. The 98 bp repeats of Mad-1 and 66 bp sequence of CY archetype strains are shown by large brackets and dotted arrows respectively. The NCCR is divided into six regions according to CY strain: Box A (36 bp), B (23 bp), C (55 bp), D (66 bp), E (18 bp) and F (69 bp). Nucleotide deletion (s) are marked by a dash (–) and nucleotide variation sites among endemic strains are marked with vertical arrows.

amplified directly from urine and blood samples of non-immunocompromized healthy tribal groups from north-eastern parts of West Bengal State of India. The objectives of the current investigation were (1) to document the prevalence or reactivation status of human polyomavirus JCV in non-immunosuppressed Indian tribal population, (2) to compare and contrast intergenic NCCR motifs of JCV variants found in healthy subjects with respect to transcription/transactivating factor binding and (3) to predict putative transcription factor binding sites within the endemic NCCR variants.

Initial infection with human polyomavirus seems to occur through the oral/respiratory route in childhood, as evidenced by detection of JCV DNA in infant tonsillar tissues, after which they remain in latent state in organs like kidneys, hematopoietic precursor cells, B-lymphocytes etc. till reactivation (Boldorini *et al*., 2003; Dorries *et al*., 1994; Monaco *et al*., 1998). Reactivation of both BKV and JCV infection with resulting urinary excretion of viruses readily occurs during conditions of immune suppression or immunocompromization. Conventional PCR has been used to detect and characterize both BKV and JCV DNA in urine of different patient groups, from different human tissues and from urine of pregnant women (Arthur *et al*., 1989; Markowitz *et al*., 1991). Regulatory regions of both BKV and JCV have been amplified and characterized from urine of bone marrow and renal transplant patients, HIV positive or negative individuals, patients with various autoimmune diseases. Full-length JCV genomes or DNA sequences have also been isolated and characterized from normal brain tissue, new born infants, immunocompetent older individuals and peripheral blood leucocytes of immunocompetent individuals (Kitamura *et al*., 1990; Elsner and Dorries, 1992, Dorries *et al*., 1994; Baksh *et al*., 2001).

Detection of JCV nucleic acid in urine (Tan *et al*., 2009; Husseiny *et al*., 2010), brain autopsy and CNS tissues (Delbue *et al*., 2008); serum/peripheral blood leukocytes (PBL) (Gu *et al*., 2003; Tan *et al*., 2009; Husseiny *et al*., 2010), bone marrow aspirates (Tan *et al*., 2009) of normal or non-immunocompromized human subjects, in different age groups, have been reported from many regions of the world. The JCV DNA occurrence in all these studies ranged from 0 to 20% based on variable sample sizes. Incidences of JCV viruria in different tribal populations showed variations with respect to the groups studied: 56 to 66% in Native Americans (Agostini *et al*., 1997), 48 to 67% in Myanmar tribals (Saruwatari *et al*., 2002) and 20 to 22% in African tribals (Chima *et al*., 1998). We have recorded a relatively high incidence of JCV DNA detection in non-immunosuppressed and healthy human tribal groups in our study (Table I). Out of 77 assayable urine and blood samples 19 (~25 %) were scored positive by gel electrophoresis and PCR product sequencing, and 58 (~75%) samples were scored negative based on the above mentioned criteria.

The non-coding control regions (NCCRs) of JC virus vary considerably among different natural isolates. Archetype like DNA, which lacks sequence repeats in the regulatory region and contains additional sequences, are generally not found in laboratory strains, but has been isolated by molecular cloning from several sources. Rearrangement of NCCRs occurs during passage in cell culture more readily. During reactivation in pregnancy, the shed viruses show archetype-like sequence rearrangement in their NCCRs (Markowitz *et al*., 1991; Markowitz *et al*., 1993). Viral strains with divergent NCCRs may have different tissue tropism and also aberrant potential for host cell transformation (Sundsfjord *et al*., 1994). It has been postulated that the rearrangement in the NCCRs may change the biological properties of polyomaviruses in the due course of a persistent infection, such as the ability to infect different target cells. Reactivation of BKV and/or JCV infection, with the resultant urinary excretion of virus, may occur in healthy individuals but occurs more frequently under conditions of immunosuppression, especially when T-cell functions are depressed (Chesters *et al*., 1983; Gardner *et al*., 1984). JCV infection has been demostrated in B-lymphocytes (Dorries *et al*., 1994) and JCV specific $CD4^+$ T-lymphocytes have been reported in healthy individuals (Gasnault *et al*., 2003). Healthy individuals have also been shown to harbour JCV-specific $CD8^+$ T-Lymphocytes, principally against $VP1_{p36}$ epitopes, in their peripheral blood mononuclear cells (Du Pasquier *et al*., 2004). Fragments of JCV DNA, but not proteins, have been detected in multiple regions of non-immunocompromized and non-PML normal brains (Perez-Liz *et al*., 2008). The authors have also hypothesized that JCV could spread within normal brain tissue through blood or infected immune cells like B-lymphocytes and remain as integrated or episomal forms within the oligodendrocytes and astrocytes till its initiation of lytic life cycle during immune impairment. Therefore this model raises the possibility of human brain being a secondary site of latency for JCV.

We have investigated the binding sites of known and reported transcription factors/transactivators in the endemic JCV NCCRs and also used computer software to search for additional transcription factors having potential to bind to this region of JCV genome. Two prominent features within the endemic JCV NCCR Box B are the absence, unlike JCV Mad-1, of full-length Purα/YB-1 binding pentanucleotide (5'-AGGGAAGGGA-3') (Chen and Khalili, 1995) and the presence of Sp1 binding site (GA Box) (5'-AGGGAGGAGC-3') (Henson *et al*., 1992) in the same

Supplementary

Table I
Predicted transcription factor binding sites (TFBS) in the NCCRs of endemic JCV strains NB1, NB2, NB3, NB6 derived using MatInspector Release Professional 8.0.5, March 2011 of Genomatix Software suite v2.5 GmbH. Selected TFBS matches are shown as alphabetically arranged vertebrate matrix families. TFBS search involved both general core promoters (0.75/Optimized) and vertebrate (0.75/Optimized) promoter element groups of MatInspector matrix family library version 8.4 (June 2011).

JCV strains (NCCRs)	Matrix Family	Detailed Family Information	Tissue	Optimized Similarity Scores	TF Binding Sequence (Core Sequence in capital)
NB1, 2, 3, 6	O$VTBP	Vertebrate TATA binding protein factor	Ubiquitous	0.90	131-gtataTATAaaaaaaag-147
NB1, 2, 3, 6	V$AP4R	AP4 and related proteins	Ubiquitous	0.92	214-gctggcAGCTggttggc-230 (–) and 486-tggccAGCTggtgaca-500 (–)
NB1, 2, 3, 6	V$BRNF	Brn POU domain factors	Brain, CNS, Endocrines System, Neuroglia, Neuron	0.89	14-tttgctgTAATtttttgct-32 (–)
NB1, 2, 3, 6	V$CAAT	CCAAT binding factors	Ubiquitous	0.81	185-ccagCCAAgcatgag-199 and 211-ggagCCAAccagctg-225
NB1, 2, 3, 6	V$E2FF	E2F-myc activator/cell cycle regulator	Ubiquitous	0.84–0.85	458-ctctgGCTCgcaaaaca-474 (–) and 456-ctggctcgcAAAAcatg-472 (–)
NB1, 2, 3, 6	V$EGRF	EGR/nerve growth factor induced protein C & related factors	Brain, CNS, Endocrine System Kidney, Nervous System, Urinoogenital System	0.88	105-ggaggcggAGGCggcct-121 and 387-gactatGGGAggggttt-403 (–)
NB1, 2, 3, 6	V$ETSF	Human and murine ETS1 factors	Hematopoietic and Immune System, Leukocytes, Lymphocytes, Monocytes	0.88–0.96	424gcacaaggGGAAgtggaaagc-444 29-caaaaaagGGAAaaacaaggg-49 140-aaaaaaagGGAAggtagggag-160
NB1, 2, 3, 6	V$FKHD	Fork head domain factors	APCs, Blood Cells, Immune System, Leukocytes, Lymphocytes	0.89	132-tatataTAAAaaaaagg-148 and 410-cacaagTAAAcaaagca-426
NB2	V$GATA	GATA binding factors	Blood and Bone Marrow Cells Hematopoietic System, Immune System, Leukocytes, Lymphocytes	0.90	199-cctaGATAtgagc-211
NB1, 2, 3, 6	V$HICF	Krueppel-like C2H2 zinc finger factors hypermethylated in cancer	Erythropoiesis, control of cell proliferation, monocyte activation	0.88	180-ggcTGCCagccaa-192 and 221-agcTGCCagccag-349
NB1, 2, 3, 6	V$HOMF	Homeodomain transcription factors	Blood and Bone Marrow Cells, Endocrine System, Hemato poietic System, Immune System, Leukocytes, Lymphocytes	0.88-0.95	13-cagcaaaaAATTactgcaa-31 and 14-tttgcagtAATTttttgct-32 (–)
NB1, 2, 3	V$IKRS	Ikaros zinc finger family	Antibody-Producing Cells, Blood Cells, Hematopoietic System, Immune System, Leukocytes, Lymphocytes	0.84	43-acaagGGAAtttc-55
NB1, 2, 3, 6	V$IRFF	Interferon regulatory factors	Antibody-Producing Cells, APCs, Blood, Bone Marrow Cells, Hematopoietic and Immune System, Leukocytes, Lymphocytes, Monocytes Myeloid Cells, Phagocytes	0.85-0.87	376-cagttatagtGAAAcccctcc-396; 429-agggGAAGtggaaagcagcca-449; and 28-gcaaaaaaggGAAAaacaagg-48
NB1, 2, 3, 6	V$MAZF	Myc associated zinc fingers	Blood Cells, Immune System, Leukocytes	0.90	387-atggGAGGggttt-399 (–)
NB1, 2, 3, 6	V$MYBL	Cellular and viral myb-like transcriptional regulators	Blood and Bone Marrow Cells, Hematopoietic and Immune System, Leukocytes Lymphocytes	0.96	370-ctaTAACtgccagtg-384 (–)
NB1, 2, 3, 6	V$MZF1	Myeloid zinc finger 1 factors	Blood and Bone Marrow Cells, Hematopoietic and Immune System, Leukocytes Myeloid Cells	0.99	428-aaGGGGaagtg-438

Table I continued

JCV strains (NCCRs)	Matrix Family	Detailed Family Information	Tissue	Optimized Similarity Scores	TF Binding Sequence (Core Sequence in capital)
NB1, 2, 3, 6	V$NEUR	NeuroD, Beta2, HLH domain	Antibody-Producing Cells, Blood and Bone Marrow Cells, Brain, CNS, Hematopoietic System, Immune System, Leukocytes, Lymphocytes, Nervous System, Neuroglia, Neurons	0.95	216-caaccaGCTGcca-228; 488-tcacCAGCtggcc-500 and 489-ggcCAGCtggtg-500 (–)
NB1, 2, 3, 6	V$NF1F	Nuclear factor 1	Brain, Central Nervous System, Digestive System, Liver, Nervous System	0.81-0.92	161-gagctggctggctGCCAgcca-191 (+); 212-gagccaaccagctGCCAgcca-348; 433-ctcCTGGctgctttccacttc-453 (–); 477-gttTTGGcttgtcaccagctg-497; 161-tggcTGGCagccagccagctc-191 (–); 165-tggcTGGCtgccagccaagca-195 (+); 433-gaagTGGAaagcagccaggag-453; 171-aaactggatggctGCCAgcca-191 (NB6); 171-tggCTGGcagccatccagttt-191 (–) (NB6); 175-tggTTGGctggcagccatcca-195 (–) (NB6); 175-tggaTGGCtgccagccaacca-195 (NB6); 355-gcccTGGCtgcatgccactgg-375; 355-ccagTGGCatgcagccagggc-375 (–); 477-cagcTGGTgacaagccaaaac-497 (–)
NB1, 2, 3, 6	V$NFKB	Nuclear factor kappa B/c-rel	Blood and Bone Marrow Cells, Hematopoietic and Immune System, Leukocytes, Myeloid Cells, Phagocytes	0.87	45-aagggaatTTCCctg-59 and 45-cagggaaaTTCCctt-59 (–)
NB6	V$NOLF	Neuron-specific olfactory factor	Antibody-Producing Cells, Blood, Bone Marrow Cells, Hematopoietic, Immune, Nervous System, Leukocytes, Lymphocytes, Neurons	0.88	460-acatgtTCCCctggctgctttcc-438 (–)
NB2, 3, 6	V$P53F	p53 tumor suppressor	Ubiquitous	0.92	441-aagcagccaggggaaCATGtttt-463 (NB6) and 452-ctctggctcgcaaaaCATGttcc-474 (–) (NB6)
NB6	V$PAX3	PAX-3 binding sites	Embryonic Structures Muscle, Skeletal Muscles	0.93	187-gagctCATGgttggctggc-202 (–)
NB6	V$PAX5	PAX-2/5/8 binding sites	Antibody-Producing Cells, Blood Cells, Endocrine System, Hematopoietic System, Immune System, Kidney, Leukocytes, Lymphocytes	0.79	177-tatgagCTCAtggttggctggcagccatc-205 (–)
NB2, 6	V$PAXH	PAX homeodomain binding sites	Brain, CNS, Endocrine System, Nervous System Neurons	0.99	16-caaaaaATTAcagca-30
NB1, 2, 3, 6	V$PLAG	Pleomorphic adenoma gene	Brain, CNS, Nervous System	0.87	223-ccaggGCTCcctctggctggcag-361 and 348-agaggGAGCcctggctgcatgcc-370
NB1, 2, 3, 6	V$PURA	Pur-alpha binds both single-stranded and douple-stranded DNA in a sequence-specific manner	Brain, CNS, Nervous System, Neuroglia, Neurons	0.97	105-ggAGGCggaggcg-117
NB1, 2, 3, 6	V$RUSH	SWI/SNF related nucleo-phosphoproteins with a RING finger DNA binding motif	Ubiquitous	0.98	410-gtttACTTgtg-420 (–)
NB1, 2, 3, 6	V$SALL	Spalt-like transcription factors	Embryonic Structures, Kidney, Urogenital System	0.96	135-atATAAaaaaaag-147
NB1, 2, 3, 6	V$SP1F	GC-Box factors SP1/GC	Ubiquitous	0.88	151-aggtagGGAGgagctgg-167 386-actatgGGAGgggtttc-402 (–)
NB6	V$TALE	TALE homeodomain class recognizing TG motifs	Bone Marrow Cells, CNS, Embryonic Structures, Hema-topoietic System, Myeloid Cells, Nervous System, Neurons	0.95	219-ctctggcTGTCagctgg-351 (–) 217-aaccagctGACAgccag-349

Table I continued

JCV strains (NCCRs)	Matrix Family	Detailed Family Information	Tissue	Optimized Similarity Scores	TF Binding Sequence (Core Sequence in capital)
NB6	V$XBBF	X-box binding factors	Antibody-Producing Cells, Blood Cells, Immune System, Leukocytes, Lymphocytes	0.90	441-aagcagccaggGGAAcatg-459
NB6	V$YY1F	Activator/repressor binding to transcription initiation site	Embryonic Structures	0.82	166-ggcagCCATccagttttagcc-186 (–)
NB1, 2, 3, 6	V$ZF02	C2H2 zinc finger transcription factors 2	Blood Cells, Immune System Leukocytes, Lymphocytes	0.87	107-ggaggccgaggCCGCctccgcct-129 (–)

JCV NB1, NB2, NB3, NB6 showed 99, 140, 144, and 155 total matches respectively. JCV NB3, 5 and 6 being identical, only NB3 was included in the search. Matrix similarity column refers to an optimized similarity match above 0.80 score while a perfect match to the matrix is marked by a maximum score of 1.00. All matches below 0.80 score were excluded in the table. A range of scores for discrete sequences are shown where appropriate. Start and end positions of TF binding sites are numbered according to the aligned NCCR sequences as shown in Figure 1. Matching sequences found in negative strands are indicated as (–); however sequences are numbered along the plus strands. Matching sequences found only in NB6 are indicated within parentheses at the end of the sequences.

region. These features are also exhibited by the archetypal CY, Tibetan LH3, Taiwanese Tai3 and the Central-North Indian IN-8 strains (Fig. 2). In the light of these findings (Chen and Khalili, 1995) the significance of partial Purα/YB-1 binding sites within the NCCRs of the endemic circulating JCV vis-a-vis the level of viral T antigen expression within the tissues needs to be validated through cell culture-based gene expression studies. The terminal two nucleotide deletions in the Purα/YB-1 binding pentanucleotide and the concomitant presence of Sp1 binding GA-Box needs to be investigated with respect to glial cell specific gene expression studies. Cellular tumour suppressor protein p53 is reported to bind to JCV large T antigen to repress viral replication (Staib *et al*., 1996) and/or directly to and regulate JCV promoter (Ariza *et al*., 1994). We have found a di-nucleotide deletion within this p53 binding site of endemic JCV strains from Oraon/Munda group but not from the Rabha group (NB6) (Fig. 2). Implications of this deletion in the NCCRs of JCV NB1 to NB5 need to be evaluated in *in vivo* studies. The predicted TFBS depicted putative binding sites for transcription factors corresponding to thirty-three matrix families (Supplementary Table I). We retained the putative TFBS matches which are reported to be active in cells/tissues such as antibody-producing cells, antigen-presenting cells, bone marrow cells, hematopoietic and immune system, leukocytes, lymphocytes, monocytes, myeloid cells, phagocytes, brain, CNS, endocrine system, kidney, nervous system, urinogenital system. Although the TFBS were common to NB1 to NB6, on a closer look we can see few transcription factors (Neuron-specific Olfactory factor or NOLF, p53 tumor suppressor, TALE homeodomain class recognizing TG motifs or TALE, X-box binding factor or XBBF and Activator/repressor binding to transcription initiation site or YY1F) matching exclusively to either positive (+) or negative (–) strands of NB6 NCCR owing to its sequence differences with that of other endemic NCCRs. While the implications of these finding cannot be guessed at this juncture, the list is likely to provide a hint for a detailed *in vivo* binding studies to validate hypotheses regarding tissue-specific promoter activities in JCV.

In summary, we have reported for the first time the NCCR architecture of the endemic JCV strains in tribal population groups from north-eastern region of India and have compared their sequences with that of other key JCV strains from Asia. The NCCR sequence analyses were done with regard to transcription factor binding to DNA sequence elements of endemic JCV NCCRs.

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Original Article

Quantification of human polyomavirus JC virus load in urine and blood samples of healthy tribal populations of North-Eastern part of West Bengal, India

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Abstract

Background: Human polyomavirus JC (JCV) is a widespread human virus with profound pathogenic potential. A study was undertaken to quantify JCV load in urine and peripheral blood samples of immunocompetent, apparently healthy tribal individuals of North-Eastern part of West Bengal, India for the first time. **Materials and Methods:** One hundred and thirteen samples of urine or blood were collected from different tribal groups of this region. For the quantitative estimation of the viral load in each sample, real-time polymerase chain reaction method using the SYBR Green dye was employed. **Results:** The viral load estimated was found in the range between 3.5×10^2 and 2.12×10^6 copies/ml of samples having a mean and median viral copy numbers of 8.67×10^5 and 9.19×10^5 copies/ml of sample respectively. **Conclusion:** The mean viral DNA load in urine samples of the studied immunocompetent population was found to be higher than that found in a study conducted in the USA, but lower than similar groups of Italy and healthy adult women in the USA. However when compared with median values of viral DNA loads in urine samples of immunocompetent human subjects of Kuwait, Portugal, and Switzerland the observed viral DNA load was found to be substantially higher.

Key words: *JC polyomavirus, North-East India, real-time polymerase chain reaction*

Introduction

JC virus (JCV) is a member of the *Polyomaviridae* family. Apart from few isolated human populations JCV appears to be widespread worldwide.[1,2] Initial infection with the virus occurs in childhood, after which the virus may persist in the kidneys and lymphoid tissues.[3] It is associated with a fatal demyelinating central nervous system (CNS) infection called progressive multifocal leukoencephalopathy (PML) in immunocompromised hosts such as in patients with AIDS[4] and involves productive infection in both oligodendrocytes and astrocytes.

The genome of the virus is a covalently closed, circular double-stranded DNA molecule having a length of about 5000 base pair.[5] It comprises of early and late coding region controlled by a common non-coding control region (NCCR). The "early" region encodes for the viral tumour antigens (T/t-antigens) and is expressed prior to initiation of DNA replication and the "late" region encodes three viral capsid proteins (VP1, VP2 and VP3).

Conventional polymerase chain reaction (PCR) has been used to detect the presence of JCV in both immunocompromised, as well as immunocompetent individuals. With the development of real-time PCR technology quantitation of viral nucleic acid loads in body fluids has become accurate.[6] However, report of quantitation of JCV load in immunocompetent subjects is scanty.

This study was done on the apparently healthy tribal groups of the North-Eastern part of West Bengal, India to estimate the quantity of viral load in individuals infected with the virus. For the quantitation of the virus, real-time PCR method was employed and oligonucleotide primers specific for the VP1 region of the viral genome was used. In our earlier study, an overall incidence rate of 25% was observed in the Oraon, Munda and Rabha tribal groups of this region.[7] However, in the present study, the incidence rate of the virus was found to be a little lower due to the incorporation of Mech population with the previously studied tribal groups and was observed to be 18.58% in all of the tribal population taken together. The viral copy number value was observed to be in the range between 3.5×10^2 and 2.12×10^6 copies/ml of sample having a mean and median copy number value of 8.67×10^5 and 9.19×10^5 copies/ml of sample respectively.

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Materials and Methods

Sample collection

The methodology was approved by our Institutional Human Ethical Committee. Urine and blood samples from 113 individuals of healthy tribal groups of the North-Eastern part of West Bengal State of India were collected with their prior informed consent. The tribal groups studied here included Oraon and Mundas of Darjeeling district, Rabha or Rava population of Cooch Behar district and Mech population of Jalpaiguri district. Samples were collected on a random basis and collection depended on the availability and willingness of the tribal subjects concerned. Three millilitre of urine specimen from all the 113 individuals were collected in Tarson 1.5 ml microfuge tubes and 1 ml of blood specimen from 53 individuals of Rava group were collected in 5 ml Tarson vials containing ethylenediaminetetraacetic acid and were brought to the laboratory in ice box and stored at −20°C refrigerator till DNA isolation.

DNA isolation, polymerase chain reaction amplification, and electrophoretic analyses

DNA isolation from both the urine and blood samples was done using the High Pure Viral Nucleic Acid Kit (Roche Diagnostic GmbH, Germany) as per the manufacturer's instruction and the isolated DNA samples were stored at −20°C refrigerator until used for quantitation of the viral load. Detection of the JCV positive samples was done primarily by normal PCR using primers specific for the amplification of NCCR of the viral genome for qualitative detection and then the quantitative approach was employed using primers specific for VP1 region. The amplified products were analysed by agarose gel electrophoresis. The Chi-square test was employed using SPSS (version 20) [IBM Corporation, New York, USA] to compare between the JCV positivity status with that with the gender of the subjects studied. For the analyses, the $P < 0.05$ were considered significant.

Standard viral DNA

Plasmid pMITC-BSMKS carrying the full length of JC viral genome (JCV Mad-1 strain; 5130 base pairs), a gift from Richard J. Frisque, Department of Biochemistry, Microbiology, Molecular and Cell Biology, Pennsylvania State University, USA was used as the standard DNA for the quantitation of the viral load by real-time PCR.

Estimation of viral load by real-time polymerase chain reaction

Estimation of the viral DNA load in 13 out of the 21 positive DNA samples isolated from both urine and blood were done with the real-time PCR method. To perform this reaction, two primers namely JCVRVF (5□-TCAATGGATGTTGCCTTTACTTT-3□) and JCVRVR (5□-ACGGGGTCCTTCCTTTCTC-3□) specific for the VP1 region of the viral genome[8] were used, which produced an amplification product of about 109 bp lengths. The specificity of the primers for JCV VP1 was checked by nr-BLAST search. The reaction was carried out in Applied Biosystems Thermal Cycler, StepOne Plus™. Each PCR reaction mixture consisted of 3 µl of both forward and reverse primers (2 pM), 10 µl of 2X SYBR Green Reagent (FastStart Universal SYBR Green Master [Rox], Roche, Germany) and 4 µl of DNA to a final volume of 20 µl. PCR protocol included the following: Initial heating at 95°C, 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 45 s. All the samples were run in duplicate. The cloned JCV Mad1 plasmid containing the whole viral genome in a 10 fold dilution series served as the standard DNA for the estimation. It was used to generate the quantification standard curve. The copy number of the plasmid DNA (pMITC-BSMKS; 8091 base pairs) was adjusted to 8.08×10^9, 8.08×10^8, 8.08×10^7, 8.08×10^6, 8.08×10^5, 8.08×10^4 copies/ml (in six tubes) following real-time PCR software, based on the concentration and the plasmid size. The sensitivity or lower detection limit of the assay was found to be 8.08×10^4 copies/ml of the plasmid. Standard curve plot is the quantity of standard viral DNA against cycle threshold (CT), where CT is defined as the first cycle in which amplification signal is detected over the mean base line. Mean base line is calculated from ΔRn values of 40 cycles.

Results

The study was carried out on apparently healthy tribal groups of North-Eastern part of West Bengal, India that included Oraon and Munda tribes from Kiran Chandra Tea Garden, Naxalbari, Darjeeling district, Rabha tribes from Poro busty, Tufangunj, Cooch Behar district and Mech tribes from Mendabari, Kalchini, Jalpaiguri district. An overall incidence rate of 18.58% was observed in the population of this region [Table 1]. Oraon and Munda groups showed a higher incidence of JCV, that is, 29.16% compared to the other groups studied. The possible association of virus prevalence with the gender of the subjects were statistically analysed. The frequency of JCV detection seems to differ between the genders and it has been found to be greater in males compared to the females in the tribes of this region with a P value of 0.003. In case of mech, due to the low positive detection of JCV (5.55%) among the random sample, the association between virus status and sex were not significant enough ($P = 0.871$). The viral load was estimated in the positive samples by real-time PCR method using primers specific for VP1 region of the JCV genome. The details regarding the copy number and CT value range of the JCV positive individuals are represented in Table 2, which also includes mean and

Table 1: Summary of the results showing incidences of the JCV in the studied tribal groups

Tribal groups	Collection site	Total number of individuals	Sex	Number of positive samples [Total (%)]	*P*	JCV positive specimen type
Oraon and Munda	Kiran Chandra Tea Garden, Naxalbari, Darjeeling district	24	Female=20, male=4	Female=03, male=04 [7 (29.16)]	0.001	Urine=7
Rabha	Poro Busty, Tufanganj, Cooch Behar district	53	Female=20, male=33	Female=01, male=11 [12 (22.64)]	0.017	Urine=2, blood=10
Mech	Mendabari, Kalchini, Jalpaiguri district	36	Female=20, male=16	Female=01, male=01 [2 (5.55)]	0.871	Urine=2
Total		113	Female=60, male=53	Female=05, male=16 [21 (18.58)]	0.003	Urine=11, blood=10

JCV=JC virus

Table 2: Details of copy number value and CT value range of JC viral DNA in both urine and blood samples of the population studied

Specimen type	CT value range	Mean CT value	Copies/ml of sample	Mean copy number	Median copy number
Urine	22.48-39.19	27.57	3.52×10^{2}-2.12×10^{6}	1.07×10^{6}	1.08×10^{6}
Blood	27.05-31.19	28.48	1.71×10^{5}-1.18×10^{6}	7.77×10^{5}	7.69×10^{5}
Total	22.48-39.19	28.20	3.52×10^{2}-2.12×10^{6}	8.67×10^{5}	9.19×10^{5}

CT=Cycle threshold, DNA=Deoxyribonucleic acid

median DNA load of the virus in both urine and blood specimen of the population studied. A wide range of viral load was found in the samples ranging from 3.52×10^{2} to 2.12×10^{6} copies/ml of sample. The mean and median copy number values were found to be 8.67×10^{5} copies/ml of sample and 9.19×10^{5} copies/ml of sample respectively.

Discussion

JC virus is widely spread in the human population[9] and is the causative agent of PML in individuals with suppressed immune system. Because of its pathogenic role in humans, a study regarding the prevalence of the virus among the normal, apparently healthy tribal groups of North-Eastern part of West Bengal, India has already been done.[7] This study aimed at quantitation of the viral load in the immunocompetent healthy tribal populations of North-Eastern part of West Bengal, India.

The North Bengal region (North-Eastern parts of West Bengal, India) is inhabited for long by several tribal groups like Santhal, Oraon, Munda, Rabha, Chik-Baraik, Toto, Koch, Mech, Bhutia and others who belong to different linguistic groups, Austro-Asiatic, Dravidian and Tibeto-Chinese.[10] Oraons are the second largest and the Mundas are the third largest tribal population after the Santhals, constituting about 14% and 7.8% respectively of the total tribal population of West Bengal State of India.[11]

Traditionally, conventional PCR have been used for the detection of the JCV DNA in all types of samples. However, estimation of viral load by conventional PCR is at best semi-quantitative. Real-time PCR method has emerged as a new tool for both detection and quantification of the viral DNA isolated from different types of specimen.[6] In this study, we have used SYBR Green dye for quantitation and primers specific for the amplification of a 109 bp fragment in VP1 region of the JCV genome.

Prevalence of JCV has been recorded in the tribal population worldwide showing variations in the incidence range of the virus in different groups such as Native Americans,[12] tribals of Africa[13] and Myanmar tribals.[14] Previously we have documented a relatively higher incidence (~25%) of JCV DNA in the urine and peripheral blood leucocytes of Oraon, Munda and Rabha tribal groups of the North-Eastern part of West Bengal, India.[7] However, the overall incidence rate of the virus was now found to be 18.58% with the inclusion of Mech population, which had lower incidences of JCV reactivation (5.55%). The association between the JCV prevalence and the gender of the subjects was studied using Chi-square test. The frequency of the virus prevalence vary between the genders and is higher in case of males (P=0.003), which is in accordance with the previous studies done on Native Americans[12] and tribals of Africa.[13] The quantitation of viral DNA load present in the body fluids viz., urine and blood of the tribal populations of this region is being reported now for the first time in this study.

We have recorded a wide range of viral copy numbers in the urine and blood samples from the population of this region, which ranged from 3.52×10^{2} to 2.12×10^{6} copies/ml of sample. Individuals infected with the virus were found to have a high amount of viral DNA load present in their urine and blood specimens. The mean (8.67×10^{5}) and the median (9.19×10^{5}) viral copy number recorded in the present study was higher

than observed in the some of the studies done earlier on healthy immunocompetent subjects. The mean (8.67×10^5 copies/ml of sample) viral load in the samples was comparatively higher than normal healthy subjects of USA having a value of only 3×10^3 copies/ml of sample.[15] The median value, 9.19×10^5 copies/ml recorded in our samples was also found to be higher compared to that of immunocompetent healthy subjects of Portugal,[16] Kuwait[17] and healthy blood donors of Switzerland.[18] However, the mean viral DNA load, if compared with observed mean values in three Italian immunocompetent subjects[19] and healthy adult women of USA,[20] was found to be lower.

We have also observed that the mean viral load in urine (1.07×10^6) was higher than that in the blood (7.77×10^5) samples of apparently healthy tribal individuals [Table 2]. The JCV is found to infect an individual in early childhood, after which it can remain in a latent state in the kidney[21] and is shed in the urine throughout the entire life without any noticeable clinical symptoms in the individual. Reactivation of JCV with the resulting urinary excretion of viruses readily occurs during conditions of immune suppression or immunocompromization. It has also been suggested that JCV could spread within normal brain tissue through blood or infected immune cells like B-lymphocytes.[22] Further studies are underway to confirm the effect of high viral load in both urine and peripheral blood leucocytes in the subsequent development of pathogenic conditions in CNS of the affected subjects.

Our study was aimed at quantitation of JCV load in body fluids of immunocompetent normal subjects. It has long been suggested that nutritional deficiency can lead to an immunodeficient condition in humans, which in turn enhances the susceptibility towards infection and disease condition.[23] Further studies are required to corroborate nutritional status of the studied tribal groups with that of the high viral load in the body fluids of the population studied.

In conclusion, the extent of the viral load in immunocompetent individuals was reported for the first time in tribal groups of this area suggesting a relatively higher amount of viral load present in the infected individuals. Given the ubiquitous nature and pathogenic potential of JCV in human subjects, further study can relate the observed viral loads in this region and their clinical implications.

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REVIEW

Entry, infection, replication, and egress of human polyomaviruses: an update

Soumen Bhattacharjee and Sutanuka Chattaraj

Abstract: Polyomaviruses (PyVs), belonging to the family *Polyomaviridae*, are a group of small, nonenveloped, double-stranded, circular DNA viruses widely distributed in the vertebrates. PyVs cause no apparent disease in adult laboratory mice but cause a wide variety of tumors when artificially inoculated into neonates or semipermissive animals. A few human PyVs, such as BK, JC, and Merkel cell PyVs, have been unequivocally linked to pathogenesis under conditions of immunosuppression. Infection is thought to occur early in life and persists for the lifespan of the host. Over evolutionary time scales, it appears that PyVs have slowly co-evolved with specific host animal lineages. Host cell surface glycoproteins and glycolipids seem to play a decisive role in the entry stage of viral infection and in channeling the virions to specific intracellular membrane-bound compartments and ultimately to the nucleus, where the genomes are replicated and packaged for release. Therefore the transport of the infecting virion or viral genome to this site of multiplication is an essential process in productive viral infection as well as in latent infection and transformation. This review summarizes the major findings related to the characterization of the nature of the interactions between PyV and host protein and their impact in host cell invasion.

Key words: polyomavirus, infection, receptor, co-receptor.

Résumé : Les polyomavirus (PyV), de la famille des *Polyomaviridae*, forment un groupe de petits virus non enveloppés à ADN bicaténaire circulaire que l'on retrouve un peu partout chez les vertébrés. Les polyomavirus n'occasionnent aucune maladie apparente chez les souris de laboratoire adultes, mais engendrent diverses tumeurs lorsqu'inoculés artificiellement chez des animaux nouveau-nés ou semi-permissifs. On tracé un lien sans équivoque entre poignée de polyomavirus humains, dont les virus BK, JC et le polyomavirus de cellules de Merkel, et une pathogenèse dans un contexte d'immunosuppression. On soupçonne que l'infection survient à un moment précoce et persiste tout au long de la vie. À l'échelle de l'évolution, il semblerait que les polyomavirus aient coévolué lentement avec des lignées hôtes animales spécifiques. Des glycoprotéines et glycolipides à la surface de la cellule hôte semblent jouer un rôle déterminant lors de l'étape infectieuse de l'entrée et dans la canalisation des virons vers des compartiments membranaires intracellulaires aboutissant au noyau, où les génomes se répliquent et sont emballés pour être relâchés. Dès lors, le transport du virion infectant ou du génome viral vers ce site de multiplication est un processus essentiel à l'infection virale productive de même qu'à l'infection latente et à la transformation. La présente revue résume les principales constatations découlant de la caractérisation de la nature des interactions entre le PyV et les protéines de l'hôte et de leur impact sur l'invasion de la cellule hôte. [Traduit par la Rédaction]

Mots-clés : polyomavirus, infection, récepteur, co-récepteur.

Introduction

Polyomaviruses (PyVs) are nonenveloped, icosahedral, double-stranded small DNA viruses that were among the first mammalian viruses to be analyzed molecularly. Comparison of viral genomes, especially the viral origin of replication, indicates that after evolving from a common ancestor this group may have diverged with their natural hosts (Soeda et al. 1980; Buck et al. 2016). Thirteen human PyVs have been identified to date. BK virus (BKPyV) was the first and JC virus (JCPyV) was the second in the group to be identified and were named after the initials of the patients from whom they were first isolated (Gardner et al. 1971; Padgett et al. 1971). Human polyomaviruses KI (KIPyV) and WU (WUPyV) were named after the institutions where they were first reported (Allander et al. 2007; Gaynor et al. 2007). Merkel cell

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polyomavirus (MCPyV) and trichodysplasia spinulosa (TS) polyomavirus (TSPyV) were named based on their discovery in Merkel cell carcinoma (MCC, a rare form of skin cancer) (Feng et al. 2008) and in TS, a rare skin condition (van der Meijden et al. 2010). Human polyomaviruses 6 and 7 (HPyV6 and HPyV7) were detected in human skin (Schowalter et al. 2010) and HPyV9 in both serum and skin (Scuda et al. 2011) and were named according to their order of discovery. Malawi polyomavirus (MWPyV) (Siebrasse et al. 2012), also known as HPyV10 (Buck et al. 2012) or Mexico polyomavirus (Yu et al. 2012), was detected in stool and skin samples roughly simultaneous. Saint Louis polyomavirus (STLPyV) was detected in a clinical stool specimen (Lim et al. 2013), HPyV12 in human liver tissue (Korup et al. 2013), and New Jersey polyomavirus (NJPyV) in endothelium of a muscle biopsy from a pancreatic transplant recipient (Mishra et al. 2014).

Classical viral infection in the host cell is initiated by interactions between viral capsid or envelope proteins and cell surface receptors. Internalization of the virion occurs either by fusion of the envelope and the plasma membrane or via endocytic pathways that lead to escape of the virion from an endocytic or other vesicular compartment into the cytosol (Smith and Helenius 2004; Marsh and Helenius 2006). Attachment to cellular receptors can directly trigger conformational changes in virus surface structures or can activate specific signaling pathways that facilitate viral entry. Replication of the viral genome, viral protein synthesis, assembly of the viral particles, and their release from the cell use the host cellular machinery. The release of the virion occurs via budding followed by exocytosis or cell lysis (Marsh and Helenius 2006). The release of nonenveloped viruses usually occurs through cell lysis, but in some cases, they may escape either by some secretary mechanisms (Altenburg et al. 1980) or may use cellular autophagy pathways for exit (Jackson et al. 2005). Recent evidences indicated that nonenveloped viruses can employ an additional escape route as a viral quasi-species population, via membrane-bound vesicles, as found in several enteroviruses, including hepatitis A virus, hepatitis E virus, bluetongue virus, coxsackievirus, rhinovirus, and poliovirus (Altan-Bonnet and Chen 2015).

Seroepidemiological surveys conducted so far throughout the world show that the seroprevalence of BKPyV, JCPyV, SV40 (Simian vacuolating virus 40), KIPyV, WUPyV, and MCPyV is high in those populations (Brown et al. 1975; Stolt et al. 2003; Kean et al. 2009). Antibodies to both BKPyV and JCPyV are present in 70%–80% of the adult population. The viruses appear to be ubiquitous, with almost no region of the world being free of anti-PyV antibodies, except in some remote populations (Brown et al. 1975). Primary infections with BKPyV and JCPyV viruses probably occur in childhood and are presumed to be subclinical (Padgett and Walker 1973; Shah et al. 1973). The viruses presumably harbor peripheral blood lymphocytes or can remain latent in the kidney (Salzman 1986; Dörries et al. 1994; Greenlee 1997). It has been hypothesized that the human PyVs probably persist indefinitely in the kidney and are reactivated and excreted in the urine during immunological impairment (Heritage et al. 1981; Arthur et al. 1986). Immunosuppression or other viral infections may also lead to reactivation of PyVs. This model of infection may be true for all human PyVs, including WUPyV and KIPyV (Heritage et al. 1981; Arthur et al. 1986; Gaynor et al. 2007; Allander et al. 2007).

In recent years, there has been a resurgence of interest in the study of the occurrence, genotype, and pathogenic associations of human PyVs, especially after the discovery of MCPyV, which has been implicated strongly in the etiology of MCC. With the growing incidences of acquired and induced immunosuppression, a thorough knowledge of PyV–host interactions may be beneficial in their control from the standpoint of developing antiviral formulations. This review encompasses the current knowledge on interactions of PyV proteins with cellular components during virion entry, infection, replication, and egress from the infected cells.

Human PyV and disease

The name “polyoma” is derived from the Greek terms *poly*, meaning many, and *oma*, meaning tumors, and these viruses were named so because they were shown to cause a wide variety of tumors when artificially inoculated into neonatal rodents (Primrose and Dimmock 1980). PyV particles have a diameter of approximately 500 Å and a sedimentation coefficient of 240 S (1 S = 0.1 ps). The capsid is made up of 360 copies of the major capsid protein VP1 arranged in 72 pentamers that are connected to the internal viral protein VP2 or VP3, the minor constituents of the viral capsid. The genome is divided into 2 major regions: an “early” region, expressed prior to the initiation of viral DNA replication, and encodes 3–4 viral tumor antigens (large, small, middle T antigens: LTAg, STAg, MTAg); and a “late” region that typically encodes 3 viral capsid proteins (VP1, VP2, and VP3). However, MCPyV virions, having native viral regulatory sequences, do not seem to express detectable amounts of VP3 capsid protein in cell culture system (Schowalter and Buck 2013). A small nonstructural protein, agnoprotein or leader protein 1, is also encoded from the late leader region in a few PyVs (SV40, BKPyV, and JCPyV); however, other characterized human PyVs do not seem to express this protein. Agnoprotein seems to have roles in viral gene expression, replication, and virus release (Gerits and Moens 2012). SV40 VP4, a newly identified late gene product, expressed very late during cell lysis, has been found to interact with experimental biological membranes and facilitate their disruption (Daniels et al. 2007; Raghava et al. 2011). A noncoding

regulatory region or noncoding control region (NCCR), comprising the viral origin of replication, is present between the early and late coding regions. The NCCR is the most variable genomic region that contains regulatory elements and usually undergoes sequence rearrangements during reactivation, thus largely influencing the outcome of the infection. Diverse deletions or duplications within the NCCRs from immunocompromised patients have been shown to augment early gene expression and rate of replication in BKPyV and JCPyV (Gosert et al. 2008, 2010).

The major capsid protein VP1 (~45 kDa) is required in the capsid assembly; the minor capsid proteins VP2 (~38 kDa) and VP3 (~27 kDa) help in the uncoating process when the viruses penetrate host cells. PyV infection in permissive cells is initiated by the binding of the virion to a receptor on the outer cell membrane. The tumor antigens (T and t antigens) are known to alter the cellular control systems and have a key role in replication, transcription of late viral genes, and assembly of virions (Brodsky and Pipas 1998).

BKPyV

The reactivation of BKPyV in immunocompromised hosts has been associated with haemorrhagic cystitis, BKV-nephropathy, and ureteral stenosis. BKPyV-associated post-transplant nephropathy is a prominent cause of renal transplant dysfunction and has become a clinical problem in allograft recipients (Hirsch 2002; Trofe et al. 2002). The typical renal histological finding of BKPyV-associated nephritis refers to scattered interstitial inflammatory cell infiltration, lysis, atrophy, necrosis of the tubular epithelium, and fibrosis of the interstitium (Drachenberg et al. 2005). Human renal proximal epithelial cells are considered to be one of the principal natural targets of BKPyV. Persistence of PyVs in patients with renal allografts may be the important cause of progressive graft dysfunction and graft loss. BKPyV persists in the urinary tract of the individual and is shed in the urine asymptomatically. BKPyV exists in 4 genotypes (I–IV) and subtypes, which are distinguishable by neutralization serology (Pastrana et al. 2013). Subtype Ia is thought to be the most prevalent subtype and is prevalent worldwide, and subtype IV is less common and its prevalence appears to vary in different world regions (Jin et al. 1993; Jin 1993; Takasaka et al. 2004).

JCPyV

JCPyV was isolated from the brain tissue of a 38-year-old man whose initials were JC (Padgett et al. 1971). JCPyV, a human neurotropic PyV, is associated with neurological diseases such as progressive multifocal leucoencephalopathy (PML). PML, a rare fatal demyelinating disease of the central nervous system, occurs in individuals with immunosuppression involving both humoral and T-cell-mediated immune response and is mainly observed in 3%–5% of HIV-infected individuals (Major 2010). It has also been observed as a late complication in patients with chronic lymphocytic leukemia, Hodgkin's disease, lymphosarcoma, and sarcoidosis (Gardner et al. 1984; Greenlee 1997). The main symptoms of PML include cognitive impairment, visual deficit, and motor dysfunction, although it may vary based on the location and size of the lesions. The role of JCPyV in malignant transformation is controversial. JCPyV was thought to exist in 5–10 or more genotypes, with Type 6 from West Africa representing the ancestral JCPyV genotype (Pavesi 2003). Several Asian subtypes have been identified and the Indian subtype has been designated as Type 2D (Cui et al. 2004). However, recent viral metagenomic analyses by Buck and others suggest that distantly related PyVs may recombine to produce new chimeric lineages, and that PyVs may have slowly co-evolved with individual host animal lineages through an established mechanism known as intrahost divergence (Buck et al. 2016). This led the group to propound a modified classification scheme of the PyVs that account for these intrahost diversifications.

MCPyV

MCPyV was detected in Merkel cell carcinoma (MCC) (Feng et al. 2008) and has been found to be present in 80%–85% of MCC tumor specimen (Dalianis and Hirsch 2013, and references therein). MCPyV has been associated with cellular transformation in highly aggressive primary cutaneous neuroendocrine skin neoplasm, termed MCC, and is considered the only human PyV to date to cause tumors in its natural host (Arora et al. 2012; Spurgeon and Lambert 2013). During detection of the virus, the LTAg was found to be truncated before the helicase domain in 80% of the cases (Duncavage et al. 2009) but maintained the ability to bind retinoblastoma protein (Neumann et al. 2011). MCPyV DNA has been detected mostly in non-melanoma skin cancers, such as in squamous cell carcinoma, basal cell carcinoma, and Bowens disease in immunosuppressed individuals (Kassem et al. 2009). Apart from detection in MCC and other diseases, MCPyV has been detected in upper (Bialasiewicz et al. 2009; Goh et al. 2009) and lower respiratory tract infections (Babakir-Mina et al. 2010).

KIPyV and WUPyV

Both KIPyV and WUPyV were discovered in the year 2007 in broncho-alveolar lavage and nasopharyngeal aspirates (Allander et al. 2007; Gaynor et al. 2007). KIPyV and WUPyV have been detected worldwide in respiratory specimens and clinical samples (Babakir-Mina et al. 2008, 2009; Kiasari et al. 2008; Bialasiewicz et al. 2008; Foulongne et al. 2008; Payungporn et al. 2008; Abed et al. 2007; Han et al. 2007; Le et al. 2007), suggesting a widespread infection, but their involvement in human disease or tumor development is not clear (Giraud et al. 2008, 2009). Reactivation and excretion of KIPyV and WUPyV have also been reported in the gastrointestinal

tract of immunosuppressed transplanted patients (Siebrasse et al. 2016) and in HIV patients.

HPyV6 and HPyV7

HPyV6 and HPyV7 were first found in the year 2010 during a study that involved isolation of full-length wild-type Merkel cell polyomavirus DNA from the skin of healthy volunteers (Schowalter et al. 2010). Both HPyV6 and HPyV7 have been detected in urine, feces, and nasopharyngeal swabs in transplant recipients (Siebrasse et al. 2012). Their relationships with a disease in immunosuppression or in malignant transformations have recently been indicated in few studies (Schrama et al. 2014; Ho et al. 2015). A novel HPyV7 strain has recently been associated with HIV- and AIDS-associated pruritic rash (Nguyen et al. 2016).

TSPyV

The intracellular presence of virus particles in a patient with trichodysplasia spinulosa (TS) was first demonstrated in 1999 (Haycox et al. 1999). Identification of the virus was not achieved until much later in plucked facial spines of a heart transplant patient with TS (van der Meijden et al. 2010). TS is a rare skin disease in immunocompromised hosts and is characterised by the development of facial follicular papules and keratotic protrusions (spicules or spines) with alopecia of the eyelashes and brows (Haycox et al. 1999; Heaphy et al. 2004; Osswald et al. 2007; Sadler et al. 2007; Lee et al. 2008; Schwieger-Briel et al. 2010). The presence and high seroprevalence of TSPyV in healthy individuals suggest a subclinical latent infection. The development of tumor in patients with TSPyV remains to be investigated.

HPyV9

HPyV9 was first discovered in the serum of a kidney transplant patient (Scuda et al. 2011) and has not been associated with any disease to date. The phylogenetic analysis of the HPyV9 genome showed higher similarity with the genome of the African green monkey-derived lymphotropic polyomavirus than with that of other PyVs.

MWPyV

MWPyV was isolated from a stool sample of a healthy child from Malawi (Siebrasse et al. 2012). In another study, Buck et al. identified a virus in a specimen from a patient with warts, hypogammaglobulinemia, infections, and myelokathexis syndrome; the virus was named HPyV10 and showed 95%–99% similarity with MWPyV, suggesting that they were the same virus (Buck et al. 2012). More recently, a human PyV named MXPyV was detected in stools of children (Yu et al. 2012), and it was found to be closely related to MWPyV and HPyV10. The association of the virus with infection in the gastrointestinal system or any other disease remains to be investigated.

STLPyV, HPyV12, and NJPyV

STLPyV was recovered from the fecal microbiota of a child in Malawi. However, its association with diarrhea is not significant. Sequence comparison with other PyVs revealed that the virus shares maximum similarity with the MWPyV (Lim et al. 2013). HPyV12 was detected in the liver as well as colon, rectum, and feces of a single individual, but its relation to the disease in humans remains to be investigated (Korup et al. 2013). The most recently discovered species, NJPyV, was isolated from the muscle biopsy of a 33-year-old pancreatic transplant recipient (Mishra et al. 2014). The name New Jersey polyomavirus was after the place from where the patient belonged.

Viral entry

The mechanism of virus entry in nonenveloped viruses can involve various mechanisms, such as disruption of the endosome at low internal pH in Adenovirus, pore formation at the plasma membrane or endosomes in Poliovirus, or entry via caveolae in SV40 (Marsh and Helenius 2006; Mudhakir and Harashima 2009).

Receptors and coreceptors

A number of cell surface receptors have been identified, of which a few act as single molecular species and a few as multiple-receptor set for viruses. Some other viruses depend on a more complex receptor, which involves interaction with at least 2 distinct plasma membrane components. Virus–host cell receptor interactions are specific and use distinct receptors and entry mechanisms to infect cells (Table 1). Glycoproteins are proteins containing oligosaccharide chains covalently attached to polypeptide side-chains, and glycolipids are lipids attached to the carbohydrate by a glycosidic bond. Gangliosides (GD1a, GD1b, GT1b, and GM1) are glycolipids, i.e., glycosphingolipids, with 1 or more sialic acids (e.g., *N*-acetylneuraminic acid, NANA) linked differentially to the sugar chain. More than 60 gangliosides are known and they differ from each other mainly by the position and number of NANA residues. Sialic acid is an N- or O-substituted derivative of neuraminic acid, a monosaccharide with a 9-carbon backbone, and is found mostly in glycoproteins and gangliosides. Glycosaminoglycans (GAGs) or mucopolysaccharides are long unbranched polysaccharides that consist of a repeating disaccharide unit consisting of an amino sugar along with uronic sugar or galactose, except for the GAG keratan.

High resolution crystal structures of truncated murine PyV (mPyV) VP1 protein, complexed with branched disialylated hexasaccharide receptor fragments, were instrumental in revealing the roles played by VP1 pentamers in contacting cellular sugar moieties. Each subunit of the VP1 pentamer has 2 antiparallel β-sheets with jelly-roll topology, and some of the loops that connect the β-strands are extensive, containing additional secondary structure elements (a small 3-stranded anti-parallel β-sheet, one α-helix, and four 3_{10}-helices) (Stehle and

Table 1. Human polyomaviruses, disease associations, cellular receptor/coreceptors, and entry mechanism.

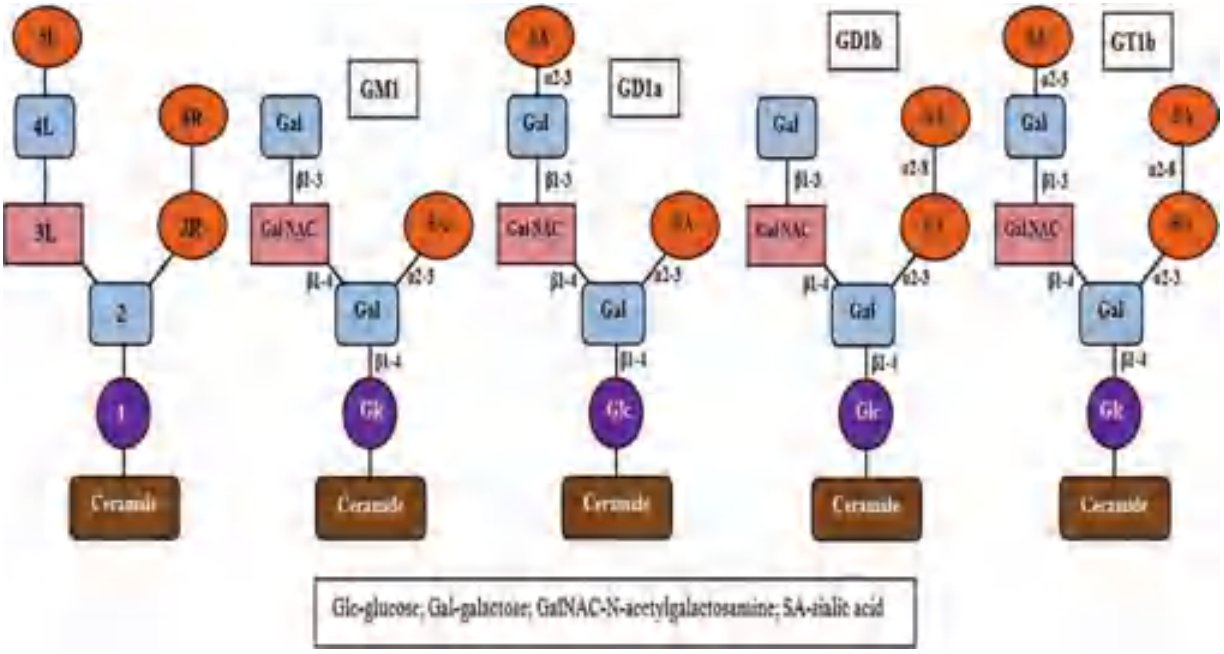
Sample No.	Virus name	Abbreviation	Disease associations	Receptor(s)/coreceptor(s) (entry mechanism)	References[a]
1	BK polyomavirus	BKPyV	Hemorrhagic cystitis and nephropathy	Gangliosides GD1b, GT1b (caveolin-mediated endocytosis)	Dugan et al. 2005; Low et al. 2006
2	JC polyomavirus	JCPyV	Progressive multifocal leucoencephalopathy	LSTc/5-HT_2 (clathrin-mediated endocytosis)	Liu et al. 1998; Assetta et al. 2013
3	KI polyomavirus	KIPyV	Respiratory symptoms (?)	Not known	—
4	WU polyomavirus	WUPyV	Respiratory symptoms (?)	Not known	—
5	Merkel cell polyomavirus	MCPyV	Merkel cell carcinoma	Heparan sulfate/sialylated glycan	Schowalter et al. 2011; Neu et al. 2012
6	Human polyomavirus 6	HPyV6	Squamous cell carcinoma (?); keratoacanthoma (?)	Not known	—
7	Human polyomavirus 7	HPyV7	Pruritic rash (?); thymoma (?)	Not known	—
8	Trichodysplasia spinulosa-associated polyomavirus	TSPyV	Trichodysplasia spinulosa	Not known	—
9	Human polyomavirus 9	HPyV9	Not known	Not known	—
10	MW polyomavirus	MWPyV	Not known	Not known	—
11	STL polyomavirus	STLPyV	Not known	Not known	—
12	Human polyomavirus 12	HPyV12	Not known	Not known	—
13	New Jersey polyomavirus	NJPyV	Vascular myopathy (?)	Not known	—

[a]References reporting receptors and coreceptors.

Harrison 1996, 1997). These studies showed that at least 3 surface grooves (pockets 1, 2, and 3) of mPyV VP1 are important in recognizing α2,3-linked α-5-*N*-acetylneuraminic acid (sialic acid, SA or NANA) on the surface of susceptible cells. In addition to binding to the usual α2,3-linked sialic acid, some mPyV strains also bind to branched oligosaccharides that carry a second α2,6-linked sialic acid. Binding of low tumorigenicity strains to the branched-chain receptors depends on the presence of glycine at position 91 on the outer surface of VP1 (Fried et al. 1981; Cahan et al. 1983; Stehle et al. 1994; Stehle and Harrison 1997). PyV VP1 residues Tyr-72, Arg-77, Gly-78, His-298, and Asn-93 were identified as essential components of pockets 1 and 2. Single mutations in these residues resulted in the complete loss of infectivity (Stehle et al. 1994; Stehle and Harrison 1997). Based on binding and flotation assays, Tsai et al. (2003) later demonstrated that specific gangliosides, GD1a and GT1b for mPyV and GM1 for SV40, can serve as plasma membrane receptors. Their model, based on the crystal structure of the VP1–oligosaccharide complex, indicated that the sialic acid-α2,3-Gal moiety on the left branch of GD1a can interact with VP1, as proposed by others (Stehle et al. 1994; Stehle and Harrison 1996, 1997) (Fig. 1). GT1b ganglioside, which has an additional attached sialic acid (residue 4R), can also bind VP1, which therefore, suggests binding of different mPyV strains equally to both GD1a and GT1b (Tsai et al. 2003). Structurally and biologically similar SV40 has also been shown to interact with GM1 gangliosides in flotation and immunoblotting assays (Tsai et al. 2003).

To investigate the linkage of sialic acid in BKPyV infection, Vero cells were treated with *Vibrio cholerae* neuraminidase or *Streptococcus pneumoniae* sialidase S and then challenged with the virus (Dugan et al. 2005). Both neuraminidase and sialidase S inhibited infections, which suggested a role for α2,3-linked sialic acid in BKPyV infections. The investigators also examined the role of specific linkages of sialic acids in infection and whether glycoproteins or gangliosides were involved in infection through complete removal of sialic acid from Vero cells. The results suggested that α2,3-linked sialic acid on an N-linked glycoprotein is sufficient to mediate infection (Dugan et al. 2005). Low and coworkers used ganglioside-containing liposomes in a binding assay with purified BKPyV and further proved that a terminal α2,8-linked disialic acid motif present in the GD1b and GT1b gangliosides plays an important role in these interactions (Low et al. 2006). Studies based on virus-like particle (VLP)-based neutralizing assays indicated that VP1 capsid proteins of each of the major BKPyV genotypes can reciprocally escape from neutralization by antibodies raised against the other types. These serotype-defining differences between the genotypes can be related to a few key amino acid differences within the VP1–BC2 surface loop (Pastrana et al. 2013). Based on cell-based transduction assays, this study also indicated that

Fig. 1. Gangliosides involved in polyomavirus binding. The general structural organization is depicted at the left. GM1, mono-sialotetrahexosylganglioside 1.

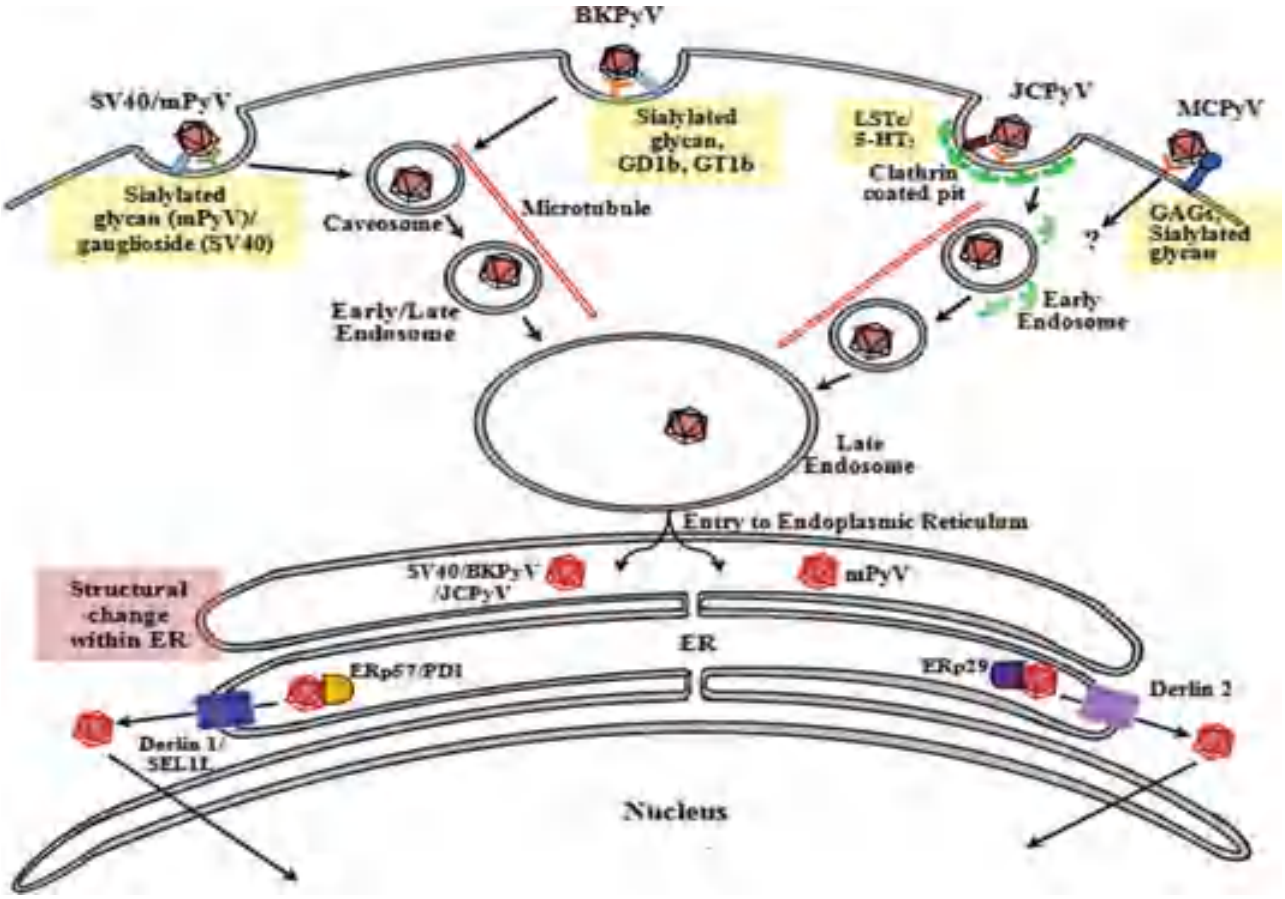


different BKPyV genotypes and subtypes may have altered attachment requirements and virulence potential in vivo, reflecting the changes in amino acids within their VP1 capsomere knob (Pastrana et al. 2013). Komagome et al. (2002) found through screening that the VLPs containing the JCPyV major viral capsid protein VP1 had the highest affinity for neoglycoproteins containing the terminal α2,6-linked sialic acid. While it was clearly established that PyV infections depend on an interaction between VP1 and sialic acids, a glycan-array screening and infectivity assay further established that JCPyV interacts with a linear sialylated pentasaccharide with the sequence NeuNAc-α2,6-Gal-β1,4-GlcNAc-β1,3-Gal-β1,4Glc (Lactoseries tetrasaccharide c or LSTc) on host glycoproteins and glycolipids (Neu et al. 2010). Conflicting results are now available with regard to the cellular entry of JCPyV. One set of experiments suggests that JCPyV internalization and cellular entry depends on the expression of serotonin receptor 5-hydroxytryptamine (5-HT) receptor 5-HT_{2A}R in the susceptible cells after attaching to sialylated sugars (Elphick et al. 2004; Assetta et al. 2013). However in vitro studies by Chapagain et al. (2007) suggested that JCPyV infection in the human brain microvascular endothelial cells is independent of the 5HT_{2A}R receptor. A subsequent study has shown that there could be a sialylated glycan-independent infectious entry pathway for both JCPyV and BKPyV, as found in heparin-like glycan-dependent MCPyV infection (Schowalter et al. 2011). The study reports complete inhibition of cellular entry of PML-mutant JCPyVs and a sialic acid nonbinding mutant BKPyV, when blocked by heparin or a heparan sulfate-blocking antibody (C.B. Buck, personal communication) (Fig. 2).

High resolution crystal structures of HPyV6 and HPyV7 VP1 revealed the presence of elongated loops that cover the bulk of the outer virion surfaces and obstruct a groove that binds sialylated glycan receptors, as in other PyVs (Ströh et al. 2014). This study could not detect interactions of VP1 with α2,3- and α2,6-linked sialic acids by nuclear magnetic resonance spectroscopy; hence, HPyV6 and HPyV7 probably engage nonsialylated cellular receptors for host cell recognition. Using a liposome flotation assay, Erickson et al. (2009) proved that the sialic acids on both the arms of GT1b are necessary for binding MCPyV VP1, but GD1a, having an α2,3-linked monosialic acid modification on the right arm, and GD1b, having no sialic acid on the left arm, do not bind to MCPyV VP1. However, sialylated glycans are not required for the initial interaction of MCPyV with the cultured human cell line but may be required as coreceptors in the entry process. GAGs, such as heparan sulfate and chondroitin sulfate, serve as primary receptors during the entry process (Schowalter et al. 2011, 2012).

Systems virology is instrumental in the global understanding of hitherto unknown virus–host interactions and especially of the dynamic nature of host responses against viruses (Bhattacharjee 2015). Therefore, this approach can discover new mutations in PyV VP1 genes, can discover the implications that these mutations have in tissue tropism and the potential for cross-species infectivity, and can also aid in the development of host-based antiviral drugs (Kilcher and Mercer 2014). Interestingly, in a recent study, an intrapatient evolution of BKPyV has been shown to produce neutralization-escape VP1 variants that may have altered tissue tropism by means of binding to a different spectrum of cell-

Fig. 2. Receptor-mediated endocytosis and intracellular trafficking to endoplasmic reticulum (ER) in polyomaviruses. Sialylated glycans, MHC I, and gangliosides on cell surfaces bind to and facilitate entry of SV40, mPyV, BKPyV, whereas JCPyV require LSTc and 5-HT_2 for its attachment and entry, respectively. MCPyV require primarily glycosaminoglycans and also gangliosides. SV40, mPyV, and BKPyV are internalized through caveosomes that traffic through the cytoplasm, using a microtubule network, to the late endosome and is finally delivered to the ER. JCPyV is internalized through clathrin-coated vesicles. Within the ER lumen, SV40, BKPvY, and JCPyV interact with ERp57 and PDI, a process required for the partial disassembly of viral capsids, and are then retrotranslocated to the cytoplasm via the ER transmembrane proteins Derlin-1 and SelL1. Murine mPyV interacts with ERp29 and requires Derlin-2 for its exit. LSTc, sialyllacto-*N*-tetraose c; GAG, glycosaminoglycan; 5-HT_2, 5-hydroxytryptamine.



surface sialylated glycan receptors, compared with the wild-type viruses (C.B. Buck, personal communication).

Receptor-mediated endocytosis

All PyVs, except JCPyV, generally enter the host cells through structures called "caveolae" present in the plasma membrane of the host (Anderson et al. 1996; Richterová et al. 2001; Pelkmans and Helenius 2003; Damm et al. 2005; Dugan et al. 2006; Parton and Simons 2007). However, SV40 can enter and infect cells by clathrin-, caveolae-, and dynamin II-independent pathways that deliver the virus via nonendosomal, cytosolic organelles to the endoplasmic reticulum (ER). This work was based on detection of an LTAg in caveolin-1-deficient postinfected mouse embryonic fibroblast and human hepatoma cell line (Damm et al. 2005).

A growing body of evidence indicates that viruses, including nonenveloped viruses, can use specific membrane microdomains or "membrane rafts" to penetrate the host cell. Caveolae are lipid-raft-rich, flask-shaped, 50–100 nm invaginations present in the plasma membranes that are stabilized by VIP-21 or caveolins (e.g., caveolin-1 or cav-1), containing mostly cholesterol and sphingolipid (Rothberg et al. 1992). Caveolin-2 is often associated with caveolin-1 in the same cell or tissue types, whereas caveolin-3 is found in skeletal and cardiac muscles. Caveolae are present at high concentrations on several cell types such as adipocytes, muscle cells, endothelial cells, but few or none are present in hepatocytes, neuronal cells, and lymphocytes. Most cell surface caveolae show limited motility and dynamics, as they are anchored by an actin cytoskeleton that plays a key role in controlling their activity and keeping them in place (Thomsen et al. 2002).

SV40 first binds to the major histocompatibility complex class I (MHC-I) antigens on the cell surface and is then trapped in the caveolae, which pinch off as a vesicle

containing caveolin-1 (Breau et al. 1992; Pelkmans et al. 2001). Subsequent internalization of the virus requires tyrosine kinases activation and dynamin II (GTPase) driven GTP hydrolysis, resulting in a signaling cascade leading to slow but efficient internalization (Oh et al. 1998; Pelkmans et al. 2002; Parton and Richards 2003). After internalization, some of the caveolar ligands are then sorted into endosomes and the Golgi complex or are transported to cav-1-positive, pH-neutral caveosomes (Pelkmans et al. 2001; Parton and Richards 2003). From the caveosomes, SV40 is transported by a microtubule-mediated process to the ER (Pelkmans et al. 2001) and then is released into the cytosol. From the cytosol, the virus particles enter the nucleus through nuclear pore complexes (NPCs) (Kasamatsu and Nakanishi 1998).

Early studies indicated that the binding of poliovirus and papillomaviruses to the cell receptors promotes conformational changes in the major capsid proteins VP1, which are then transmitted to the other capsid proteins (Magnuson et al. 2005; Schelhaas et al. 2007). This conformational change results in the exposure of hydrophobic sequences at the viral capsid surface that may participate in membrane penetration. These conformational changes in the viral capsid proteins could be detected by changes in their sensitivity to proteases or in the antigenic properties of the viral proteins (Fricks and Hogles 1990; Selinka et al. 2003). The recognition of sialic acid moieties on host cell membrane proteins induces conformational changes in both major and minor capsid proteins, as evidenced by resistance to protease (e.g., trypsin) digestion by the VP1-SA complexes, which is probably required for binding to entry receptors and internalization, thus is crucial for productive infection in mPyV (Cavaldesi et al. 2004).

Kinetics and mechanisms of BKPyV entry have been studied in the monkey kidney cell line Vero to investigate the roles of clathrin-dependent or caveola-dependent endocytosis (Eash et al. 2004; Eash and Atwood 2005). Several important findings emanated from these studies: virus internalization is slow and occurs between 2 and 4 h postinfection, is independent of clathrin-coated pit, is dependent on an intact caveolin-1 scaffolding domain and microtubule network, is sensitive to tyrosine kinase inhibition, and requires membrane cholesterol. Similar results were observed in the study of BKPyV entry into human renal proximal tubular epithelial cells (Moriyama et al. 2007). The JCPyV virus enters into the target cells via clathrin-mediated endocytosis and is dependent on actin polymerization and proper assembly of clathrin-coated pits; this entry is comparatively faster than the entry kinetics of SV40 (Pho et al. 2000). After entry into the cells, the virus localizes to the early and late endosomes and is hydrolyzed within the lysosome; therefore, unlike SV40, a low pH environment within the lysosome is critical for productive JCPyV infection (Ashok and Atwood 2003). Cytoplasmic transport of JCPyV in glial cells depends on microtubules and intermediate filaments, as evidenced by the sensitivity of productive infection to cytochalasin D, nocodazole, and acrylamide (Ashok and Atwood 2003). Their data suggested that microtubules may be involved either directly with the viral proteins or through a dynactin-independent dynein family member, or microtubules may be required for transport of intermediate filament-associated proteins and hence for proper assembly of intermediate filaments facilitating JCPyV transport to the nucleus (Ashok and Atwood 2003). While it was known that the engagement of SV40 to cell surface receptors can induce tyrosine kinase-mediated phosphorylation of caveolin-1 (Dangoria et al. 1996), JCPyV binding to human glial cells induces an intracellular signal that triggers downstream phosphorylation or activation of the MAPKs ERK1 and ERK2, and the viral entry is dependent on involvement of the endocytic protein Eps15 (Querbes et al. 2004).

MCPyV is a member of a divergent clade of PyVs that lack the conserved VP3 N-terminal motif. Schowalter and Buck (2013) also showed that VP3 is neither detectable in MCPyV-infected cells nor found in native MCPyV virions. Experimental mutation of possible alternative VP3-initiating methionine codons did not significantly affect MCPyV infectivity in culture, and VP2 knockout resulted in a 100-fold decrease in native MCPyV infectivity. Their pseudovirus-based experiments confirmed that VP2 plays an essential role in the infection of some cells but not in all. Their work also showed that myristoylation of the N terminus of VP2 was important for its function.

ER-mediated viral trafficking

Complex membranous networks within the host cell, such as the plasma, endolysosomal, and ER membranes, because of their specialized ER-to-cytosol retro-translocation machinery, have an important role in the initiation and progression of viral infections (Inoue and Tsai 2013). PyVs are the only viruses known to penetrate the ER membrane during entry into the host (Tsai et al. 2003; Inoue and Tsai 2013). Earlier electron microscopic studies revealed that the virus enters into host cells as small vesicles and are then targeted to the ER (Mattern et al. 1966; Mackay and Consigli 1976; Kartenbeck et al. 1989). Upon arrival onto the ER, SV40 is released into the ER lumen from GM1, presumably through a release process involving ER resident proteins (Inoue and Tsai 2011).

The ER-localized SV40 is large and intact and contains the minor capsid proteins VP2 and VP3 and the genome. The intact viral particle then penetrates the ER membrane to reach the cytosol where it disassembles (Inoue and Tsai 2011). During penetration of the ER, the virus first undergoes host-triggered conformational changes and then crosses the ER membrane, and finally is released into the cytosol (Inoue and Tsai 2013). Conformational changes in the PyV capsid, the disassembly process, and transport of its genetic material from ER to the cytosol require the participation of several putative

ER resident proteins. ER resident redox-active protein disulfide isomerase family members (PDI, ERp57, and ERp72) can isomerize or reduce the virus disulfide bonds and can play an important role in destabilizing the PyV VP1 disulfide bond network (Inoue and Tsai 2013). However, the precise combination of PDI members engaging a specific PyV family member may differ because of subtle differences in the viral disulfide bond arrangements. In some PyVs, a dimeric redox-inactive PDI protein (ERp29) untangles the VP1 carboxy-terminal arms exposing hydrophobic VP2 and VP3 to generate a hydrophobic viral particle (Magnuson et al. 2005; Rainey-Barger et al. 2007). The hydrophobic virus is probably maintained in a soluble state by binding to the Hsp70 chaperone BiP in a reaction regulated by the J domain containing co-chaperone ERdj3 (Goodwin et al. 2011).

Next, the hydrophobic virus integrates into the ER membrane to initiate membrane penetration, where ER-associated degradation (ERAD) membrane components (Derlin-1, Derlin-2, Sel1L, RMA1, BAP29, and (or) BAP31) have been proposed to facilitate PyV's entry, at least indirectly, into the cytosol (Inoue and Tsai 2013 and references therein). Although not well-understood, the release of virions into the cytosol may depend on the ER membrane J-proteins (DNAJ B12, B14, and C18) that stimulate the binding between the virus and cytosolic Hsp70 (Inoue and Tsai 2013). The SV40 capsid proteins VP2 and VP3 have been shown to play an important role in the ER escape (putatively through viroporin) and are regulated by their interaction with VP1 major capsid protein (Daniels et al. 2006*a*, 2006*b*). ERp29, structurally related to PDI protein (Mkrtchian et al. 1998), acts as a factor in inducing a conformational change in PyV particles by exposing the C-terminus of VP1 protein without the complete disintegration of the virion structure (Magnuson et al. 2005). Portions of VP2 and VP3 are also exposed that mediate the binding to the ER membrane, although only VP2 can perforate the experimental model membranes (Rainey-Barger et al. 2007).

The ERAD pathway during BKPyV intracellular trafficking has been studied in renal proximal tubule epithelial (RPTE) cells using proteasome and ERAD inhibitors (Jiang et al. 2009; Bennett et al. 2013). Transfection of the dominant-negative ER-transmembrane proteins Derlin-1 and Derlin-2 in RPTE cells and glutathione *S*-transferase pull-down assays revealed the involvement of Derlin-1, but not Derlin-2, in BKPyV escape from the ER to the cytosol (Jiang et al. 2009; Bennett et al. 2013). Proteasome function was also indicated in successful BKPyV trafficking, like SV40, in proteasome inhibition assays (Bennett et al. 2013).

Nuclear entry and replication

Nuclear entry

Nuclear transport of molecules less than 9 nm in diameter or of proteins smaller than 40 kDa occurs through the aqueous channels of the NPC by passive diffusion, whereas transport of larger molecules (>45 kDa) is mediated by facilitated nuclear transport that requires signals on both the molecule to be transported and on the cytoplasmic receptors (Pante and Kann 2002 and references therein; Wente and Rout 2010).

Nucleocytoplasmic transport of cargo >45 kDa depends on transport factors belonging to the family of karyopherin β proteins (e.g., importin β (IMPβ) and importin α (IMPα)). The β-karyopherins that mediate nuclear import are generally known as importins (IMPs), whereas those mediating nuclear export are known as exportins (EXPs). The IMPαs are adaptors that function as heterodimers with IMPβ1 in nuclear import, while IMPβs can mediate transport in either direction through the NPC (Fulcher and Jans 2011). IMPs associate with their macromolecular cargo in the cytoplasm, either directly or indirectly via adaptor proteins. They dock to components of the nucleoporins and translocate to the opposite side of the nuclear envelope to release their cargo. The cargo release is achieved by association of the IMPs with the GTPase Ran in the GTP-bound form (RanGTP). In nuclear export, cargo recognition occurs in the nucleus in the presence of RanGTP, and the complex dissociates in the cytoplasm upon GTP hydrolysis. A variety of strategies have been employed by viruses for the transport of their genomic cargo, including disassembly at the NPC, disruption of the nuclear envelope, or may involve both of them (Fay and Panté 2015). The nuclear entry and exit of cargo protein involves (*i*) the NPC on the nuclear membrane and (*ii*) recognition of nuclear localization sequences (NLSs) on the proteins to be imported by specific IMPs and EXPs (Lange et al. 2010; Wente and Rout 2010; Marfori et al. 2011; Fulcher and Jans 2011).

To enter the nucleus via interaction with the host's nuclear import machinery, SV40 must expose NLSs that are internal in the virion architecture. SV40 may escape from the ER to the cytoplasm and then enter the nucleus through the NPC, or it may enter the nucleus directly from the ER by penetrating the inner nuclear membrane; the available data favors the former possibility (Cohen et al. 2011). SV40 particles undergo a partial disassembly in the ER, exposing the internal capsid proteins VP2 and VP3. The partially disassembled SV40 particles retain some of the SV40 capsid proteins (VP1, VP2, and VP3) in addition to the viral genome (Norkin et al. 2002). For nuclear protein import, the best characterized nucleotransport signal is the classical NLS (cNLS), which consists of either 1 (monopartite) or 2 (bipartite) stretches of basic amino acids. Monopartite cNLSs are exemplified by the SV40 LTAg NLS (^{126}PKKKRRV^{132}) (reviewed in Lange et al. 2007). Accessibility and enhancement of nuclear transport depend on phosphorylation-mediated modulation of the NLS and the nuclear export signal of the protein cargo. In the SV40 LTAg, protein kinase CK2 phosphorylation increases the affinity of recognition of the

NLS by IMPα1 or IMPβ1, thereby accelerating the nuclear import rate, which can be further enhanced by phosphorylation by double-stranded DNA-dependent protein kinase (dsDNA-PK) at specific sites (Fulcher and Jans 2011). The SV40 virion may enter the nucleus through the NPC through the interactions between NLSs at the common C-terminal basic amino acids of VP2 and VP3 (VP1 and VP2 NLSs) and the canonical nuclear import machinery (Nakanishi et al. 2002, 2007). Anti-IMPα and anti-IMPβ antibody-based co-immunoprecipitation assays with SV40 DNA postinfection in permissive cells indicated that minor capsid proteins must associate with virion DNA for nuclear entry (Nakanishi et al. 2002, 2007). Only a small fraction of virions that expose an NLS deliver their genomes into the nucleus; however, a complete dissociation of the virion within the cell cytoplasm prevented the nuclear entry of the viral DNA (Nakanishi et al. 2002, 2007). Kuksin and Norkin (2012) reported that VP2 and VP3 NLSs may function only in targeting SV40 disassembly intermediates to the nucleus, but SV40 genomes dissociate from VP2 and VP3 before or at the point of nuclear entry and thus enter the nucleus devoid of these viral proteins. However, contrasting views on nuclear entry of SV40 have been provided by a study which indicated that viral infection leads to a breakdown of the nuclear lamina and disruption of the nuclear envelope, allowing the virus to pass directly from the ER lumen to the nucleus (Butin-Israeli et al. 2011).

Since BKPyV VP2 and VP3 share homology with that of SV40 at the basic C-terminal amino acids important for nuclear localization, elegant site-directed BKPyV VP2 and VP3 mutation and drug-based inhibition of IMPβ in RPTE cells have been designed to ascertain the commonality of nuclear entry mechanism (Bennett et al. 2015). This study could show that specific lysines in VP2 and VP3 are important for localization of BKPyV to the nucleus and that VP2K319T and VP3K200T mutants were defective in nuclear localization. The importance of the major capsid protein VP1 was reiterated, as in SV40, by showing that expression of this protein in transfected cells enabled nuclear transportation of mutated minor proteins (VP2K319T and VP3K200T). It was also shown in an ivermectin-based inhibition assay that the inhibition of IMPα and IMPβ largely affected early gene expression of BKPyV in RPTE cells. In an earlier study by Qu et al. (2004), the nuclear entry mechanism in JCPyV was studied that used a FITC-labeled JCV VLP in infection of HeLa (nonpermissive) and SVG (permissive) cells. The postinfection fluorescence study showed that nuclear entry of NLS-deleted VLPs was markedly inhibited in HeLa and SVG cells, highlighting the importance of VP1 NLS in the nuclear translocation of VLPs. This study also indicated the importance of both IMPα and IMPβ in nuclear entry by JCPyV VLPs (Fig. 3). Nuclear sequestration of human endogenous hVam6p (involved in cytoplasmic lysosomal trafficking) by the MCPyV LTAg has been reported, which may help in MCPyV replication and egress (Liu et al. 2011).

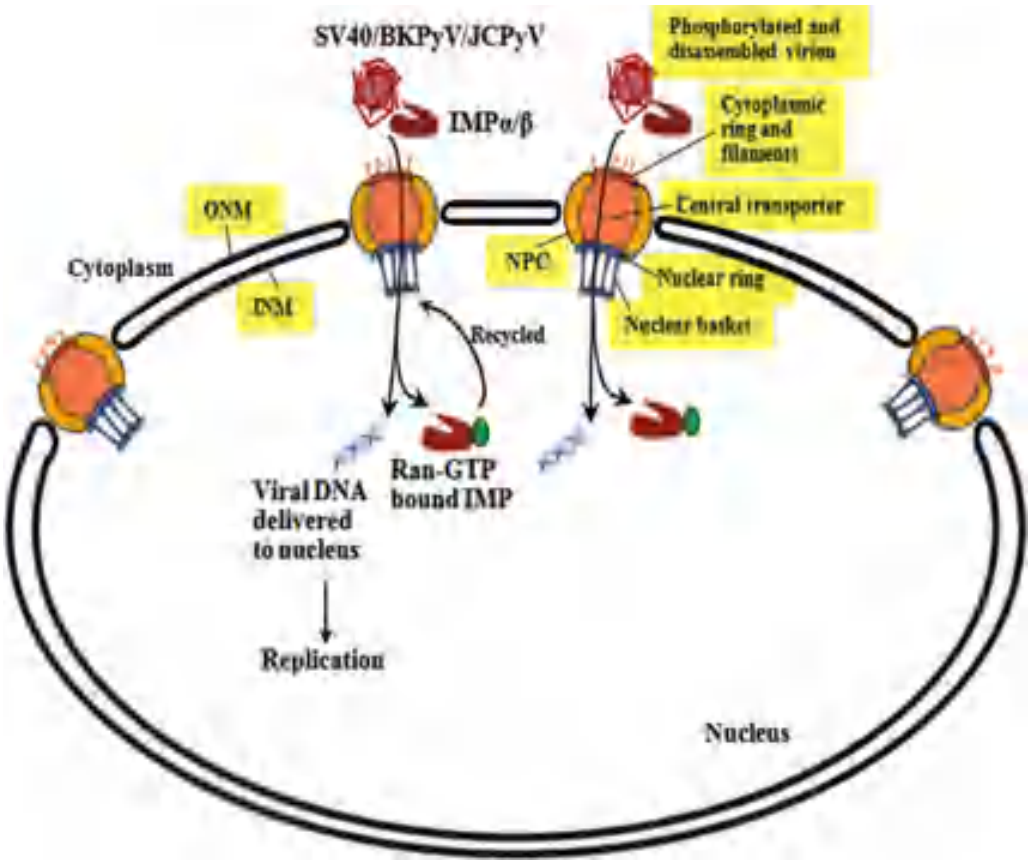
DNA replication

The replication of PyV minichromosomes has been historically used as a model system to study the mechanisms of cellular DNA replication (Kelly et al. 1988; Challberg and Kelly 1989; Stillman 1989; Hurwitz et al. 1990). There are controversial views with regard to the resemblance of in vitro replication of PyV and the host DNA replication (Fanning et al. 2009). Recent findings indicate that PyV minichromosomes undergo multiple rounds of replication and there is a dispensability of DNA polymerase ε (epsilon) (Fanning et al. 2009). Additionally, it was found that at least SV40 and mPyV induce an ATM-mediated DNA damage response in infected cells, which might contrarily arrest normal cell cycle. Present knowledge indicates that SV40 and mPyV manipulate host DNA damage signaling and repair pathways, in a largely unknown manner, to control viral minichromosome replication (Fanning et al. 2009). In the MCPyV-infected cells, components of the ATM- and ATR-mediated DNA damage response pathways were found to accumulate in the MCPyV LTAg-positive nuclear foci, suggesting an important role of DNA damage response in viral DNA replication (Tsang et al. 2014).

The in vitro study of SV40 replication has been instrumental in establishing fundamental processes of initiation of eukaryotic DNA replication (Bullock 1997). The replication of SV40 or mPyV DNA requires the viral origin of DNA replication and only 1 virus-encoded protein, the large T-antigen (LTAg) to replicate the viral minichromosome. The other necessary 40 proteins, a set of 10 human cellular proteins, are supplied by the host cells (Waga and Stillman 1994; Bruckner et al. 1995; Bullock 1997). In JCPyV, Hsp70 interacts with VP2 and VP3 and LTAg, and accumulates heavily in the nucleus of the infected cells. An association of VP2 and VP3 with LTAg through their DNA binding domains was also observed; this association enhances LTAg DNA binding to Ori resulting in the induction of viral DNA replication (Saribas et al. 2014).

The SV40 origin of replication is a 64 bp segment of the viral genome (extends between nucleotides 5211 and 31), which contains all the sequence elements required for in vivo and in vitro DNA replication (Deb et al. 1986, 1987; Challberg and Kelly 1989; Bullock 1997). The arrangement of the SV40 and mPyV core origin is very similar to the core origin domains of BKPyV and JCPyV. The SV40 core origin contains 3 functional regions: (*i*) an early palindrome (EP); (*ii*) 4 GAGGC pentanucleotide sequences, collectively referred to as the pentanucleotide palindrome (PEN) or binding site; and (*iii*) a 17 bp AT-rich domain. These 3 domains coordinate the binding, melting, and unwinding activities of LTAg (Borowiec et al. 1990; Bullock 1997). The 71 bp MCPyV replication origin was shown to include a poly(T)-rich tract and 8 variably

Fig. 3. Mechanism of nuclear entry in SV40, BKPyV, and JCPyV. ONM, outer nuclear membrane; INM, inner nuclear membrane; NPC, nuclear pore complex; IMP, importin.



oriented GAGGC-like pentanucleotide sequences, of which 4 are required for the interaction of the MCPyV LTAg (Kwun et al. 2009). The entire origin of MCPyV contains 10 potential LTAg binding sequences (PS1–PS10), and the optimal initiation of replication requires both the viral STAg expression and intact cellular Hsc70 binding to support efficient viral DNA replication (Kwun et al. 2009).

The SV40 LTAg plays a central role in the initiation of viral DNA replication by means of binding to 4 pentamer repeats (PEN) of core origin. Once bound to the origin, the LTAg assembles into a head-to-head double hexamer in the presence of ATP. Binding of LTAg causes a significant change in the local DNA structure at the viral replication origin (Kelly et al. 1988; Challberg and Kelly 1989; Stillman 1989; Borowiec et al. 1990; Bullock 1997). Initially, 2 LTAg DNA-binding domains (OBD; amino acids 131 to –259) bind to each half of the palindromic sites of the origin and then promote assembly of 8 additional LTAg monomers with the concomitant binding of ATP and ADP with the LTAg AAA+ (ATP associated and ATP binding) domain. Then the duplex DNA at the origin melts through its interaction with β-hairpin motifs of the AAA+ domain of LTAg (Gai et al. 2004; Reese et al. 2006). The remaining 4 OBDs of the LTAg hexamer interact with the already formed 2 OBD–PEN complexes to form an open spiral, and ultimately each hexamer surrounds 1 strand and displaces the other strand of the origin DNA leading to helicase structure (Reese et al. 2006; Fanning et al. 2009). The role of J domain of viral LTAg, either directly or indirectly, seems to be a requisite in viral DNA replication, at least in vivo (Bullock 1997; Sullivan and Pipas 2002). The ATPase activity of LTAg is functionally related to its helicase activity and both the activities are present in the same molecule (Kelly et al. 1988). However, LTAg-mediated unwinding reaction requires the presence of additional cellular proteins, such as single-strand DNA-binding protein or replication protein A (RPA) and topoisomerase I and II (Jiang et al. 2006).

Both LTAg and STAg antigens are required for MCPyV-positive MCC cell survival and proliferation (Houben et al. 2012). In enhancing the LTAg-dependent replication, the MCPyV STAg targets the cellular SCF (complex of Skp1, Cul1, and F-Box protein) ubiquitin ligase protein complex SCF^{Fbw7}, where Fbw7 has been shown to be dysregulated or mutated in several cancers and to be important in diverse signaling pathways regulating cell proliferation and tumor suppression (reviewed in Kwun et al. 2013). The MCPyV STAg enhances genome replication by promoting accumulation of the LTAg through the inhibition of Fbw7 (Kwun et al. 2013). This targeting of SCF^{Fbw7} is mediated through an LT-stabilization domain

(LSD) of STAg. Mutation in STAg LSD abolishes stabilization of LTAg, eliminates cell transformation, inhibits viral replication and induction of several cellular oncoproteins, including c-Myc and cyclin E.

In the next step, DNA polymerase α-primase (pol-prim) synthesizes RNA primers at the origin of DNA replication (Stillman 1989; Bruckner et al. 1995; Fanning et al. 2009). Experimental evidence indicates that at least 2 subunits of pol-prim form specific complex with helicase domain of each SV40 LTAg hexamer (Challberg and Kelly 1989; Stillman 1989; Huang et al. 1998). The synthesis of nascent RNA primer for DNA synthesis seems to be dependent on the concomitant binding of LTAg to both pol-prim and RPA (Fanning et al. 2009). This event probably leads to compact binding of RPA with the single-stranded DNA, which allows pol-prim to gain access to the DNA template for primer synthesis. Similar RPA–ssDNA binding remodeling by LTAg may lead to primer extension by pol-prim and also the switch to DNA polymerase δ, replication factor C, proliferating cell nuclear antigen (Fanning et al. 2009).

Agnoprotein enhances the DNA binding activity of LTAg to the viral origin (Ori). Phenylalanine residues are known to have critical roles in protein–protein interaction, protein folding, and stability. Mutation of all the 3 phenylalanine residues present in the agnoprotein resulted in inefficient replication of mutant virus (Saribas et al. 2012).

Viral assembly and egress

Assembly of virions occurs either in the cytosol or in the nucleus of a host cell and involves polymerization of the capsid protein-like viral minichromosomes (Garber et al. 1980). The release of nonenveloped virus usually occurs through cell lysis, but in some cases, they may escape either by some secretary mechanisms (Altenburg et al. 1980) or use cellular autophagy pathways for exit (Jackson et al. 2005). During maturation, changes in viral chromatin composition can be observed that may include an increase in the level of H3 and H4 histone, acetylation in previrion and mature virions, degradation of H1 histones, and association of non-histone proteins with mature extracellular virion chromatin complex (Salzman 1986).

The SV40 late genes encode viral structural proteins, which remain silent during the early phase of infection. Viral structural proteins are gradually added to the viral DNA–histone complex to form immature virions, which subsequently mature to give rise to extracellular mature virions (Fanning and Baumgartner 1980; Salzman 1986). In vitro studies with expressed VP1 protein of mPyV, JCPyV, and BKPyV revealed that it has the capability of self-assembly into virus-like particles that are composed of 72 capsomeres that include only pentamers arranged in a $T = 7$ surface lattice (Montross et al. 1991; Chang et al. 1997; Li et al. 2002). These VLPs had the same antigenicity as that of natural viral empty capsids (Li et al. 2002). Heterologous in vivo expression studies showed that the VP1 capsid protein expressed in *Saccharomyces cerevisiae* and cultured insect cells retained native antigenic as well as sialic acid-binding capacities (Hale et al. 2002). Disulfide bonds are also found to have an important role in maintaining the integrity of the human PyV VP1 structure by protecting calcium ions from chelation (Chen et al. 2001). In a study of the DNA-binding capacity of JCPyV VP2 it was shown that the last 13 amino acids, most importantly Lys (332) and Lys (336), within the DNA-binding domain of VP2 were essential for this binding (Huang et al. 2001). The host chaperone system, including Hsp70, has an important role in the viral assembly process, as it binds to VP1 post-translation. Chromy et al. (2003) showed that Hsp70 chaperones interact with the C-terminal domain of VP1 to inhibit calcium-mediated capsid assembly in vitro, and both prokaryotic and eukaryotic Hsp70 chaperones can assemble VP1 capsomeres in vitro into uniform capsids through an energy-dependent reaction. Sucrose gradient sedimentation and cesium chloride gradient ultracentrifugation analyses of the mature virions of agnoprotein-negative JCPyV revealed that they could assemble into irregularly sized virions and that agnoprotein alters the efficiency of formation of VP1 VLPs. Further analyses showed that some fractions of agnoprotein colocalize with VP1 in the nucleus, suggesting its role in virion assembly (Suzuki et al. 2012). Some of the viruses assemble at specific intracellular locations, often termed "virus factories" that represent subcellular scaffolds, where genomes and capsid proteins are brought together for assembly into virion particles (Erickson et al. 2012). The site for nuclear virus assembly factories is promyelocytic leukemia nuclear bodies (PML-NBs). In JCPyV, the major and minor capsid proteins cooperatively accumulate in the nuclear domain 10 (ND10), also known as PML-NBs, where they assemble into virion particles (Shishido-Hara et al. 2004).

The process of release of PyV from the host cell has been studied earlier by Clayson et al. (1989), where the workers observed that the escape mechanism of SV40 does not involve cell lysis and may be released from the apical surfaces of the infected cells. Similarly, nonlytic release mechanism has recently been observed in BKPyV (Evans et al. 2015). It was also demonstrated by the group that ~1% of total infectious virus progeny is released into the media of cultured RPTE cells by 48 h post-infection and that this egress route can be inhibited by an anion channel blocker (DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) known to affect cellular secretion pathways. However, recent evidence indicates that late agnoprotein (JCPyV) or VP4 (SV40) can act as "viroporin" and facilitate plasma membrane permeabilization or disruption and thus enhancing the viral release (Suzuki et al. 2010; Raghava et al. 2011).

Summary and concluding remarks

In the last few decades, extensive research on the functions of the individual viral proteins in the host cell and that on their interactions with cellular signaling pathways has been instrumental in discovering hitherto unknown virus–host interactions and especially of the dynamic nature of host responses in viral pathogenesis. Initial interaction between a virus and a host cell is mediated by attachment of the viral surface glycoproteins to the host cell surface attachment factors, which can be either a glycolipid or a glycoprotein. While mPyV recognizes gangliosides GD1a and GT1b and SV40 recognizes GM1, BKPyV utilizes α2,3-linked sialic acid of a glycoprotein and (or) α2-8-linked disialic acid motif of the GD1b and GT1b gangliosides (glycolipid) for interacting with host cell plasma membrane. JCPyV recognizes a linear sialylated pentasaccharide (LSTc) on host cell glycoproteins and glycolipids or an α2-6-linked sialic acid on glycoprotein for its attachment to host cells. JCPyV has also been shown to bind additionally to a 5-HT_2 family of serotonin receptors. However, sialylated glycans (glycolipid and glycoprotein) are not required for initial attachment of MCPyV virions to susceptible cells but may be required as coreceptors in the entry process. Glycosaminoglycans, such as heparan sulfate and chondroitin sulfate, may serve as initial attachment receptors during MCPyV infectious entry to keratinocytes or melanocytes.

Internalization of the virion occurs either by fusion of the envelope and the plasma membrane or via an endocytic pathway and then escapes the endocytic compartment (ER) and enters the cytosol. Caveolae-mediated internalization occurs in all human PyVs except JCPyV, which employs clathrin-mediated endocytosis. Cellular transport of the virus cargo to the ER depends on a microtubule network. Upon arrival on to the ER, viruses are released into the ER lumen, presumably through a release process involving ER resident proteins. The unique environment within the ER directs specific structural rearrangements in the viral capsid proteins like VP2 and VP3, by a process that involves PDI ERp57 (BKPyV, JCPyV, and SV40) or ERp29 (mPyV). The structurally modified virus then engages different retrotranslocons depending on Derlin-1 and Sel1L (BKPyV, JCPyV, and SV40) or Derlin-2 (mPyV) for their escape to the cytosol. This is followed by further capsid rearrangements and subsequent engagement of the NPC for delivering the genome to the nucleus. Nucleocytoplasmic transport of the viral cargo depends on IMPβ and IMPα. IMPs associate with their macromolecular cargo in the cytoplasm via the mediation of adaptor proteins. They dock to components of the nucleoporins and translocate to the opposite side of the nuclear envelope to release their cargo for viral genome replication and packaging. Subsequent to DNA replication and chromatin maturation within the host cell nucleus, virus capsid assembly is initiated. The host chaperone system, including Hsp70, has an important role in the viral assembly process that involves an important role played by agnoprotein, at least in JCPyV. The release of PyVs (e.g., BKPyV) from cells has been reported to be nonlytic in nature and may bud off from apical surfaces of the infected cells. Viroporin-mediated lytic release of JCPyV is also indicated by a few other studies.

PyV VP1 surface-loop variations and key mutations at specific sites appear to be the determinants in initial interactions of this capsid protein in the infectious entry process. Intrapatient mutational variations can also be speculated to be key events in modifying tissue tropism, virulence, and subsequent infections. Genetic and protein-level variations existing among the different genera of this virus group, and their interactions with keys proteins of the host cell, can be used in designing a framework for a treatment regime, especially in viruses that have proven pathogenic potential in human. A thorough knowledge regarding virus–host interactions may be instrumental in developing host-centric drug targets, thus avoiding viral resistance.

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RESEARCH Open Access

Aloe vera gel homogenate shows anti-inflammatory activity through lysosomal membrane stabilization and downregulation of TNF-α and Cox-2 gene expressions in inflammatory arthritic animals

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Abstract

Background: *Aloe vera* leaf gel has proven efficacious roles in the amelioration of several human diseases and illness-conditions. Specific purified gel-derived bio-constituents as well as the naturally harvested unprocessed *A. vera* gel have shown promise in modifying systemic inflammation. However, the synergistic role of natural herbal remedies, a mainstay of traditional Indian Ayurveda, has not been evaluated rigorously in this plant. In this study, the prevention of membrane lysis and protein denaturation in the presence of *A. vera* gel homogenate up to the concentration of 1000 μg/ml of gel has been assessed in vitro. Also, regulation of expression of inflammation-mediator genes (TNF-α and Cox-2) has been investigated in vivo in Freund's complete adjuvant (FCA)-induced inflammatory arthritic Wistar albino rats in a 28-day long study following the daily oral supplementation of *Aloe vera* gel homogenate doses up to 0.40 and 0.80 g/kg body weight (low-dose and high-dose groups respectively).

Results: Our results indicated that *A. vera* gel homogenate inhibits hypotonicity-induced (74.89 ± 1.26%) and heat-induced (20.86 ± 0.77%) RBC membrane lyses respectively at a concentration of 1000 μg/ml, compared to indomethacin standard (80.52 ± 0.65% and 43.98 ± 1.52% respectively at 200 μg/ml concentration). The similar concentration of gel also showed 39.35 ± 4.25% inhibition of protein denaturation compared to standard diclofenac sodium (46.74 ± 1.84% at 100 μg/ml concentration) in vitro. When assessed in vivo, TNF-α expression was found to be decreased by 35.88% and 38.52%, and Cox-2 expression was found to be decreased by 31.65% and 34.96%, in low-dose and high-dose groups respectively, when compared to the arthritic controls.

Conclusions: Our findings justify the role of unprocessed *A. vera* gel homogenate in preventing tissue damage and in the downregulation of TNF-α and Cox-2 gene expressions for the immune-modulation of inflammatory arthritis condition.

Keywords: *Aloe vera* gel, Membrane stabilization assay, TNF-α, Cox-2, Relative expression, RTqPCR

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Background

Inflammation is a common complex biological cascade of events that occurs in response to any kind of injuries, mechanical damage, infection, toxin exposure, or chemical irritation in the tissue. Inflammatory pathways lead to the protection of the body. However, prolonged inflammation can cause chronic disorders and extensive tissue damage. Depending on the duration of the inflammatory responses in the body, the inflammatory processes are commonly grouped into acute (short-duration) and chronic (long-duration) inflammation. During inflammation, immune cells release lysosomal contents that act as mediators of inflammation. These enzymes work non-specifically on the nearby cells and cause further damage by breaking their membranes. This process significantly increases the degree of inflammation [1]. The erythrocyte (RBC) membranes are considered as a model of the lysosomal membrane for their structural similarity [2]. It is expected that the drugs or products capable of preventing the rupture of the erythrocyte membrane experimentally would also protect the lysosomal membrane of the affected tissue in vivo resulting in the inhibition of inflammation. On the other hand, an increase in protein denaturation has been a hallmark of inflammation [3]. Drugs exhibiting the protein denaturation-inhibition in vitro are expected to elicit similar results in biological systems and would stabilize inflammation [4].

Rheumatoid arthritis (RA) is a complex systemic inflammatory disease of the bone joints which leads to disabilities of joint movements. Presently, about 0.5–2% of the world population is affected by the disease. The current treatment of RA focuses on the pain reduction and inhibition of disease manifestation through blockage of prime mediator molecules related to the disease [5]. Cytokines and Cox-2 are the prime regulators of inflammatory diseases. TNF-α is one of the important cytokines which regulates the progression of inflammatory RA by inducing different signaling pathways. These signaling pathways are instrumental in immune cell infiltration and increased production of other cytokines [6, 7]. Elevated COX-2 expression in synovial tissues of the arthritic joint is mediated chiefly by the pro-inflammatory cytokines TNF-α and IL-1. The effector product of Cox-2 is prostaglandin E2, which contributes extensively to the degree of inflammation [8, 9].

To treat the consequences of extensive inflammation and inflammatory diseases like RA, non-steroidal anti-inflammatory drugs (NSAIDs), or steroids are commonly used. But due to the different side effects of conventional NSAIDs and steroids, the use of herbal remedies is increasingly becoming a popular choice. However, many of these herbal remedies lack scientific exploration and thus experimental validation [10]. These products are generally classified as complementary and alternative medicines (CAMs). CAMs are generally inexpensive and with no or low side effects. In the Indian CAM system, Ayurveda emphasizes the use of plant and animal products, dietary supplements, minerals, and salts, largely in crude and unprocessed forms, in amelioration of disease conditions. World Health Organization (WHO) has documented that about 80% of the world population directly or indirectly depends on ethnic medications [10, 11].

Aloe vera (Family Xanthorrhoeaceae) is one of the pioneer plants which have been used historically to reduce inflammatory symptoms by different ethnic populations. Researchers have found potent wound healing, anti-inflammatory, anti-arthritic, and anti-nociceptive properties of the *Aloe vera* gel in appropriate model systems [5, 12–14]. The interaction of the plant gel constituents with inflammation modulators is a key area of interest at the experimental level [15]. There is no detailed work reported on such naturally occurring unprocessed *A. vera* gel in animal models and thus the presented data is expected to contribute to the existing body of knowledge on the medicinal properties of *A. vera* gel. In the present work, assessment of in vitro anti-inflammatory potentials of unprocessed naturally harvested *A. vera* plant gel homogenate is done and in vivo assessment of the expression of two apex biomolecules of inflammation namely TNF-α and Cox-2 has been done in FCA-induced inflammatory arthritic rat model following *A. vera* gel homogenate oral consumption.

Methods

Collection of plant materials and authentication

Naturally grown *A. vera* plants were collected from Siliguri and adjacent regions which is located in the sub-Himalayan Terai region of West Bengal, India. Collection of the plant material and experiments were done during the year 2016–2018. The plant was identified by competent authority, and a voucher specimen was deposited [Accession No. 09884].

Preparations of plant extract and dose determination

Crude *Aloe* gel was collected by peeling out the green outer dermal layer and by taking out the gel aseptically with the help of a scalpel. For the in vitro anti-inflammatory tests, the gel was weighed and properly homogenized with isotonic buffer solution or with distilled water (for hypotonicity-induced membrane stabilization test) to obtain final concentrations of 600, 800, and 1000 μg of gel/ml. The sample was freshly prepared every time before use. Authors postulate that the process of simple homogenization preserves all the ingredients of the crude gel in the same proportion as it is obtained naturally from the leaves. The use of homogenization as an extraction process has been supported by other works [16, 17]. For the in vivo experiments on arthritic rat models, doses of 0.40 and 0.80 g of *Aloe vera* gel/kg body weight (b.w.)

(low dose or LD and high dose or HD groups respectively) were prepared by homogenizing *Aloe vera* gel with distilled water at a ratio of 1:3 (w/v). These doses were prepared based on our previous works [5, 18] considering the possible amount of daily consumption of the *Aloe vera* gel to be 25–50 g for a human weighing 60 kg. No post-harvesting extraction or processing was done to preserve the naturally available bioactive components of the gel.

Drugs and chemicals

All the chemicals used for the experiments were molecular biology grade chemicals procured from Merck, Sigma Aldrich and HiMedia (India). Freund's complete adjuvant was procured from Sigma, USA. Reverse transcriptase enzyme was obtained from Thermo Fischer, USA; total RNA was prepared using Trizol (Invitrogen, USA); random hexamer, oligo dT, and dNTP were used from GCC Biotech, India, and SybrGreen reaction mixture was obtained from Roche, USA.

Experimental animal maintenance

Wistar albino male rats (*Rattus norvegicus*) (150 ± 15 g) were procured from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) registered animal vendor (M/s Chakraborty Enterprise, Kolkata, India) after the approval of the experimental protocols by the Institutional Animal Ethical Committee (IAEC, NBU) (Registration number 840/ac/04/CPCSEA; IAEC protocol number IAEC/NBU/2018/02). The animals were housed in the departmental animal house facility at 24 ± 2 °C with a 12 h/12 h day/night cycle and were fed with standard pellet and water was provided ad libitum. All animals were acclimatized for a period of at least 10 days before the initiation of the experiments. Four or fewer rats were kept per cage during the entire period.

In vitro anti-inflammatory tests

Collection of blood and preparation of erythrocyte suspension for in vitro membrane-stabilizing experiments

Three milliliters (3 ml) of fresh blood was collected in an EDTA vial from a healthy human volunteer who had not taken any NSAID at least 15 days prior to the collection. Erythrocyte suspension was prepared following the protocol described by Anosike et al. [2] and was stored at 4 °C prior to the experiments [2].

The membrane stabilizing activities of *A. vera* gel homogenate were assessed by a method following Shinde et al. [19] with some minor modifications.

Hypotonic solution-induced hemolysis test

Hypotonic solution-induced hemolysis test was undertaken following the methodology of Shinde et al. [19], with some modifications. *Aloe vera* gel homogenates were prepared to attain final concentrations of 600, 800, and 1000 μg of gel/ml either in hypotonic solution (homogenized in distilled water) or in isotonic solution (homogenized in isotonic buffer solution of pH 7.4). The RBC membrane breakage in hypotonic situation was calculated compared to that in the isotonic solution. In standard group, standard drug indomethacin was added to 5 ml isotonic buffer as well as to hypotonic distilled water in separate tubes to attain a final concentration of 200 μg/ml [19]. The absorbance values (OD) of the supernatants were measured at 540 nm. The percentage of inhibition of hemolysis was calculated considering the hemolysis occurring in the hypotonic solution or in distilled water of the control tubes to be 100%. Thus, the percentage of inhibition (PI) of hemolysis was calculated using the following equation:

$$\mathrm{PI} = \left[1 - \left(\frac{\mathrm{OD2} - \mathrm{OD1}}{\mathrm{OD3} - \mathrm{OD1}}\right)\right] \times 100$$

where:

OD1 = absorbance of test sample in isotonic solution

OD2 = absorbance of test sample in hypotonic solution (distilled water)

OD3 = absorbance of control sample in hypotonic solution (distilled water)

Heat-induced hemolysis test

For this test, one untreated control group, three experimental plant extract-treated groups, and one standard drug group were considered following the methodology described by Shinde et al. [19] with some minor modifications. In the experimental groups, *Aloe vera* gel was homogenized in isotonic phosphate buffer solution (pH 7.4) at a final concentration of 600, 800, and 1000 μg/ml. The standard drug group contained indomethacin in 5 ml of isotonic buffer solution at a final concentration 200 μg/ml [19]. Absorbance values (OD) of supernatant was measured at 540 nm. The percent inhibition (PI) of hemolysis was calculated using the following equation [20]:

$$\mathrm{PI} = \frac{(\mathrm{OD2} - \mathrm{OD1})}{\mathrm{OD2}} \times 100$$

where:

OD1 = absorbance of heated test sample (isotonic buffer)

OD2 = absorbance of heated control sample (distilled water)

Protein denaturation-inhibition test

The protein denaturation-inhibition test was done following established protocol [21] with some minor modifications to investigate the protein denaturation-inhibition activity of *Aloe* gel homogenate. For the experiment, along with 0.2 ml of egg albumin and 2.8 ml of PBS (pH 6.4), 2 ml of various concentrations of *Aloe vera* gel homogenates were added to experimental groups

which finally gave rise to 600, 800, and 1000 μg/ml concentration of gel in the groups. Diclofenac sodium was used as standard drug. In the standard drug group, the drug was added in the mixture to achieve a final concentration of 100 μg/ml in the 5 ml mixture which contained 0.2 ml of egg albumin and 2.8 ml of PBS (pH 6.4) [21]. The absorbance of the solutions (OD) was measured spectrophotometrically at 660 nm. The percentage of inhibition (PI) of protein denaturation was calculated using the following equation [22]:

$$\mathrm{PI} = \frac{(\mathrm{OD2} - \mathrm{OD1})}{\mathrm{OD2}} \times 100$$

where:
OD1 = absorbance of heated test sample
OD2 = absorbance of heated control sample

In vivo anti-arthritic tests

The experimental set up consisted of 24 rats distributed in four animal groups each containing 6 rats (n = 6). Animals were randomly distributed in one positive control (PC), one FCA or negative control group (FCA), and two experimental groups of LD and HD respectively, receiving daily oral supplement of *A. vera* gel homogenate at a dose of 0.40 g/kg b.w. and 0.80 g/kg b.w. respectively, once a day from the first day of the experiment till 28th day. Freund's complete adjuvant (Sigma, USA) was administrated (0.1 ml) in the right hind paw of all the experimental rats except PC on the first day. Arthritic swelling was observed within 3–4 days and a booster dose of same amount was given on 14th day. The inflammatory paw swelling of all the groups was observed and was assessed through the measurement of paw circumference with the help of a vernier caliper up to 28th day at a regular interval of 3 days (data not shown). The animals were sacrificed on 28th day following complete anesthesia using diethyl ether. On the day of sacrifice, blood was collected separately from each rat. Total RNA was prepared using Trizol (Invitrogen, USA) following the instruction of the manufacturer. The total RNA prepared separately from each of the animals of each group was then pooled together group-wise. From each group, sufficient amount of cDNA was prepared using reverse-transcriptase enzyme (Thermo Fischer, USA), random hexamer, oligo dT, and dNTP (GCC biotech, India) following the protocol suggested by the manufacturer (Thermo Fischer, USA). The prepared cDNA was used to assess the relative expression of TNF-α and Cox-2 among different groups in Lightcycler 96 real-time quantitative PCR (Roche, Switzerland). The exon-specific primers of TNF-α and Cox-2 was used along with a housekeeping gene GAPDH as endogenous control (Table 1). In brief, 5 μl of cDNA was used and mixed with 10 μl of SybrGreen, 0.3 μl of each of the forward and reverse primers (10 mM) and 1 μl of dNTP. Reaction volume was adjusted up to 20 μl by adding nuclease-free water. Annealing temperature of 59 °C was standardized for all the three genes and 45 amplification cycles were implemented. The relative mRNA expressions of the selected genes were calculated using $2^{-\Delta\Delta Ct}$ method. In this method, the expression of target genes were measured in fold change values which were analyzed with respect to the endogenous control gene expression.

Statistical analysis

The in vitro anti-inflammatory tests were carried out in triplicates for each of the groups. All statistical analyses for anti-inflammatory tests were done using GraphPad prism ver 6.01. All the data were represented as mean ± S.E.M. (standard error mean) and were analyzed with one-way ANOVA followed by Dunnett's multiple comparisons test. The results were considered statistically significant at $P < 0.05$ compared to the control group. The *** denotes significance value at $P < 0.001$.

Results

In vitro anti-inflammatory tests

Effect of Aloe vera gel homogenate on hypotonicity-induced hemolysis of hRBCs

The standard NSAID drug indomethacin showed the highest protection (80.52 ± 0.65%) at the concentration of 200 μg/ml against hypotonicity-induced lysis of RBCs. *A. vera* gel homogenate protected the human RBCs in a concentration-dependent manner (Fig. 1a). Among the experimental groups, highest protective effect was seen in 1000 μg/ml *A. vera* gel homogenate dose group (74.89 ± 1.26%).

Effect of Aloe vera gel homogenate on heat-induced hemolysis of hRBCs

All the doses of *A. vera* crude gel homogenate (600, 800, 1000 μg/ml) showed a significant inhibition activity against

Table 1 List of the primers used in the real time quantitative PCR method

Gene	Forward primer	Reverse primer
GAPDH	ATGACTCTACCCACGGCAAG	CTGGAAGATGGTGATGGGTT
TNF-α	AGCCCTGGTATGAGCCCATGTA	CCGGACTCCGTGATGTCTAAGT
Cox-2	TGTATGCTACCATCTGGCTTCGG	GTTTGGAACAGTCGCTCGTCATC

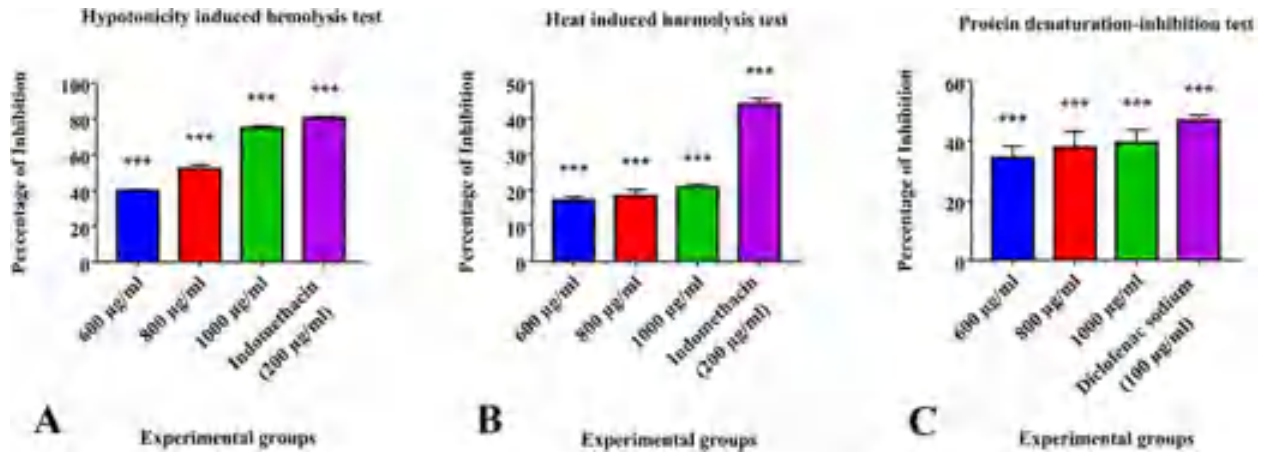

Fig. 1 Results of in vitro anti-inflammatory tests in graph. Hypotonicity-induced hemolysis test of hRBC (**a**), heat-induced hemolysis test of hRBC (**b**), and protein denaturation-inhibition test of hen's albumin (**c**) when treated with *Aloe vera* gel homogenate. All data represents % of inhibition ± SEM. Significance value at $P < 0.001$ is indicated as ***. Indomethacin and diclofenac sodium are used as standard drugs in experiments as indicated. Control group (not shown in the figures) is considered to exert 100% hemolysis

heat-induced hemolysis of hRBCs (Fig. 1b). The PI of hemolysis within experimental groups was dose dependent and maximum inhibition of hemolysis was observed in 1000 µg/ml dose group (20.86 ± 0.77%). Standard NSAID drug indomethacin showed the maximum inhibition of 43.98 ± 1.52% at 200 µg/ml concentration.

Effect of Aloe vera gel homogenate on protein denaturation-inhibition test

All the dose groups of *A. vera* gel homogenate showed significant inhibition of protein denaturation in a dose dependent manner (Fig. 1c). In the experimental groups, *Aloe* dose groups of 600 µg/ml, 800 µg/ml, and 1000 µg/ml showed 34.27 ± 3.86%, 37.82 ± 5.30%, and 39.35 ± 4.25% inhibition of protein denaturation, respectively. The standard NSAID drug diclofenac sodium showed an inhibition of 46.74 ± 1.84% at a concentration of 100 µg/ml.

In vivo anti-arthritic test

The expression of the target and the internal control genes were assessed in the negative control (FCA) and experimental animals (LD and HD) relative to the normal animals (PC) (Figs. 2 and 3). For both the genes, inhibition of expression of target genes in the experimental groups was observed in dose-dependent manner. The TNF-α showed an elevated expression (fold change) in the FCA group (2.271 ± 0.85) compared to PC (1 fold) (Fig. 3a). TNF-α expression was reduced in both the *Aloe* gel-fed groups (1.456 ± 0.11 and 1.396 ± 0.10 folds in LD and HD groups respectively). Cox-2 increased in FCA-treated animals compared to positive control (1.842 ± 0.68) but decreased in the dose groups (1.259 ± 0.11 and 1.198 ± 0.083 folds for LD and HD groups respectively) (Fig. 3b). It is evident that the expression of TNF-α was decreased by 35.88% and 38.52% in LD and HD groups respectively compared to FCA group animals; Cox-2 expression decreased by 31.65% and 34.96% respectively in LD and HD compared to FCA group animals.

Discussion

Erythrocyte membrane stability test is a well-established study to screen the possible anti-inflammatory effect of synthetic drugs as well as of various traditional herbal extracts [19]. During inflammation, lysis of the membranes of the lysosomal vesicles occurs that releases their component enzymes which induce the inflammatory response. Thus, a stabilized membrane prevents the release of its contents as well as the progression of inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) exert their beneficial effects by either stabilizing the lysosomal membranes or by inhibiting the release of lysosomal enzymes [2]. Exposure of RBCs to hypotonic medium or high temperatures results in the lysis of the RBC membranes accompanied by hemolysis and oxidation of hemoglobin. In the hypotonic solution, the hemolytic effect is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Increased body temperatures also cause the rupture of the RBC membrane resulting in hemolysis [23]. In our study, a dose-dependent relationship of *Aloe* gel homogenate against both hypotonicity and heat-induced hemolysis has been found. Therefore, both the doses of the plant extract may inhibit the release of lysosomal content during the inflammatory processes. A possible explanation for the membrane stabilizing activity of plant extracts could be an increase in surface area/

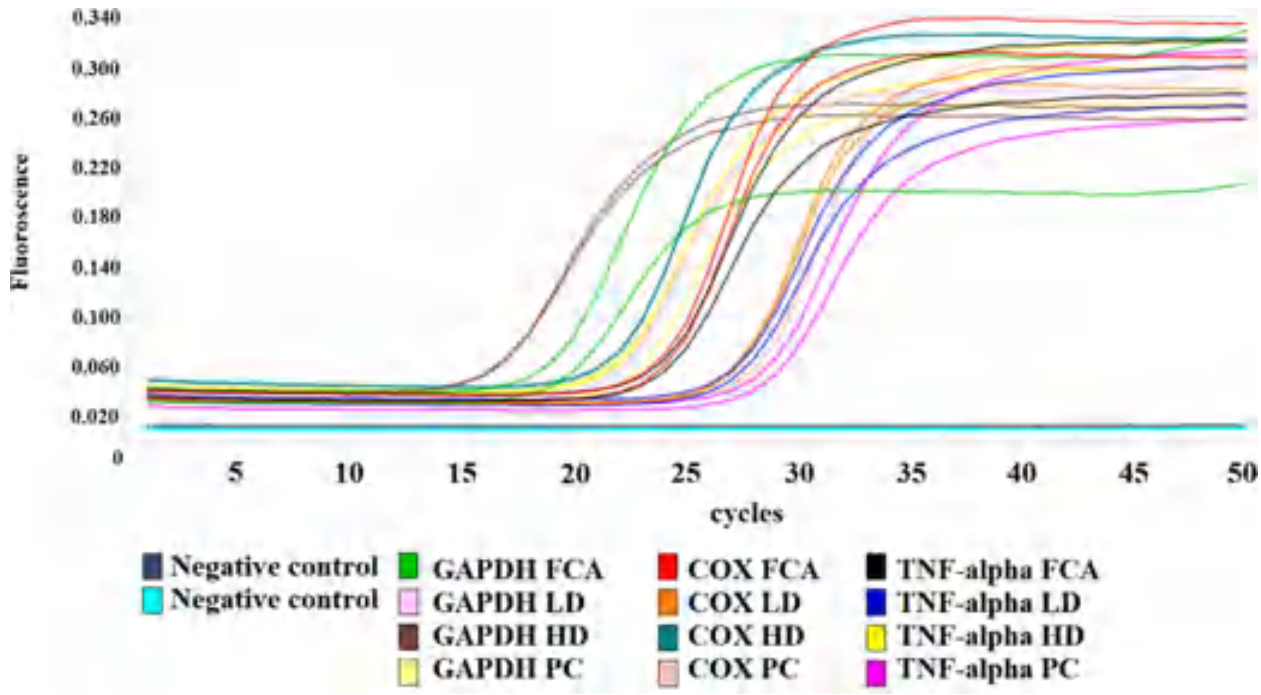

Fig. 2 Amplification curves for all the three selected genes in different experimental groups (in duplicates) in reverse transcription quantitative PCR

volume ratio of the cell [24] or by a stabilization of the skeletal proteins such as tropomyosin [25, 26].

In the protein denaturation-inhibition test, the denaturation of egg albumin was induced by heat treatment. Heat exposure causes the breakage of the functional three-dimensional structure of protein. Protein denaturation is a well-documented feature in chronic inflammatory diseases like RA, especially in the severe stages of the disease [27]. Standard NSAID drugs can inhibit heat-induced protein denaturation [4, 21]. In this study, it has been found that *Aloe* gel homogenate can also inhibit heat-induced protein denaturation. This signifies the anti-inflammatory roles of the plant in vitro and further encourages exploration of its efficacy in inflammatory conditions in vivo.

Cytokines are the key mediators of inflammation. TNF-α is a pro-inflammatory cytokine released from macrophages and monocytes. TNF-α is responsible for the transportation of more immune cells and chemokines to the inflamed region. It also initiates the production of matrix metalloproteinases responsible for cartilage degradation [7]. On the other hand, Cox-2, an immune

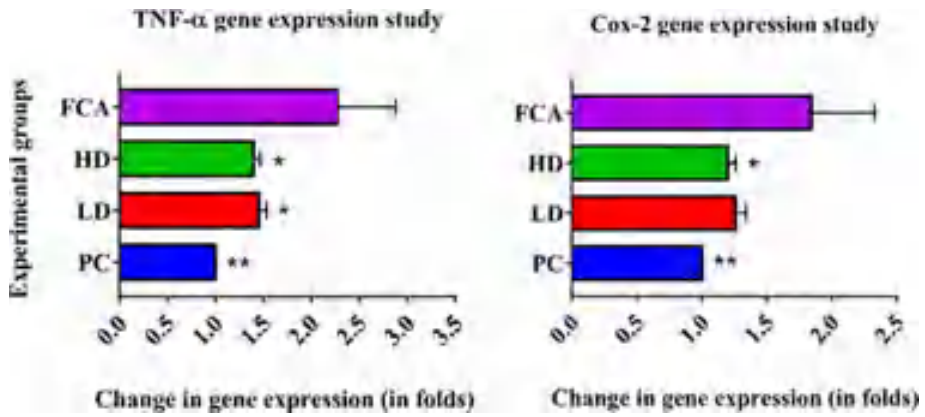

Fig. 3 Results of in vivo anti-inflammatory tests in graph. Relative expression fold changes in the TNF-α (**a**) and Cox-2 (**b**) genes assessed through reverse transcriptase real-time quantitative PCR. The significance value of low dose (LD) and high dose (HD) groups are compared with the experimental group (FCA). Positive control (PC). *P* value at $P < 0.01$ is considered significant

modulator, is responsible for the increased production of prostaglandin E2 which results in increased pain and swelling at the site of inflammation [9]. Cytokines along with Cox-2 are target bio-molecules of the host body which have a regulatory role on inflammation progression. NSAIDs primarily work on cyclooxygenase pathway [28]. Cytokine inhibitors have been introduced in the medication against inflammation as well.

In the present study, our results show that the *A. vera* unprocessed gel homogenate downregulates TNF-α as well as Cox-2 expressions in experimental animals in a dose-dependent manner. The crude unprocessed homogenized form of *A. vera* gel has been orally administered without any post-harvesting processing, a method which is in traditional use in different ethnic communities of Egypt, Rome, Africa, and Asia [29]. In our previous works, we have reported the ameliorative role of unprocessed *A. vera* gel homogenate in the regulation of inflammatory and arthritic symptoms in experimental rats. The paw circumference, serum biochemical parameters, and blood profile were restored to normal levels after oral feeding of unprocessed *Aloe* gel homogenate [5, 30]. The present study now further shows that the unprocessed *A. vera* gel homogenate concomitantly downregulates both TNF-α and Cox-2 in the experimental animal groups. This report can be considered as baseline data describing the efficacy of crude unprocessed plant products against inflammatory arthritis. By not extracting the gel in polar or non-polar solvents, we presume, the natural constituents of *A. vera* gel have been restored in its natural proportion and thus natural synergistic role of the gel has been monitored. Considering the previous works done in this regard, different bioactive compounds of the gel has already been mentioned by other workers which contribute to the efficacy of the plant as an anti-inflammatory resource. Davis et al. [12] has mentioned that the polysaccharides obtained from *Aloe vera* are potent anti-inflammatory agents; Hutter et al. [31] identified 8-[C-beta-D-[2-O-(E)-cinnamoyl]glucopyranosyl]-2-[(R)-2-hydroxypropyl]-7-methoxy-5-methylchromone from *Aloe barbadensis* (Synonym *Aloe vera*) as anti-arthritic agent. Apart from these findings, anthroquinones were identified as potent anti-inflammatory mediators in *Aloe vera* by Kashirsagar and co-workers [15]. The TNF-α downregulating activity of the plant has also been documented by Prabjone et al. [32] in helicobacter pylori-infected rats. It is highly expected that the anti-inflammatory properties of the subject plant in model systems will be equally attributed to the human system as well. Our data showed that the crude *A. vera* gel reduced the extent of lysosomal membrane lysis and protein denaturation in vitro and also regulated the expressions of major pro-inflammatory cytokine TNF-α and a key inflammation modulator Cox-2 in vivo following daily oral consumption in inflammatory RA rat models. To our knowledge, it is the first report on the effect of unprocessed *Aloe vera* gel oral treatment on TNF-α and Cox-2 gene expressions in arthritic animal models.

Conclusions

The study validates the efficacy of unprocessed aqueous *Aloe* gel homogenate in inflammatory disease condition. Our data indicate promising anti-inflammatory activity of *Aloe* gel in the inhibition of lysosomal membrane lysis, protein denaturation in vitro, and downregulation of TNF-α and Cox-2 expression in vivo. The scientific basis of use of raw unprocessed *A. vera* thus has been explored. However, an expression study on a broader spectrum of cytokines would further clarify the scenario.

Abbreviations

A. vera: *Aloe vera*; RA: Rheumatoid arthritis; NSAIDs: Non-steroidal anti-inflammatory drugs; CAMs: Complementary and alternative medicines; WHO: World Health Organization; LD: Low dose; HD: High dose; b.w.: Body weight; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; IAEC: Institutional Animal Ethical Committee; ANOVA: One-way analysis of variance; EDTA: Ethylenediaminetetraacetic acid

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Plant authentication

The identification of the plant species was authenticated by the Department of Botany, University of North Bengal, India, and a voucher specimen was deposited in the Departmental herbarium of the Department of Botany, University of North Bengal bearing the Accession No NBU-09884.

Authors' contributions

S.P, D.M, D.N, A.S, and J.R performed the in vitro experiments, analyzed the data, and prepared the tables and figures; S.P, D.M, and S.C performed the in vivo experiments and prepared and analyzed the data. S.P, D.M, and S.C also prepared the manuscript. T.K.C and S.B contributed to the concept and design of the experiments and data analyses, contributed to the critical revision of the manuscript, and gave the final approval of the manuscript for publication.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The work was approved by the Institutional Animal Ethical Committee of the University of North Bengal (IAEC, NBU) (Approval number 840/ac/04/CPCSEA).

Consent for publication

Not applicable.

Competing interests

Authors declare no conflict of interest.

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