行政院國家科學委員會專題研究計畫　成果報告

人類多瘤性病毒 JCV 與人類腫瘤相關性之研究

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ABSTRACT

Human polyomavirus JCV can infect oligodendrocytes and results in human progressive multifocal leukoencephalopathy. As with infected cells that are non-lytic, JCV exhibits transforming ability. The efficient JCV large tumor antigen (Tag) may trigger cellular signaling resulted in cellular transformation and uncontrolled proliferation in vitro or animal studies. Recent reports provided that presence of JCV DNA or mRNA of JCV Tag has been demonstrated in human central nervous system (CNS) neoplasms of different histopathologic types. However, the association with JCV and human CNS neoplasms was still unclear. We collected 36 CNS neoplasms compared with 7 non-neoplastic CNS lesions by using a nested polymerase chain reaction method for identifying viral DNA and immunohistochemistry for viral protein expression. Of 36 CNS neoplasms, 18 primary CNS neoplasms contained the Human polyomavirus DNA, JCV DNA. The tumor types included astrocytoma, medulloblastoma/PNET, schwannoma and meningioma. In contrast, 7 non-neoplastic CNS lesions all identified Human polyomavirus sequence including 7 JCV and 1 BKV viral DNA. There were 1 case with coexisting presence of JCV and BKV DNA. The detection rate of JCV was higher than BKV in CNS neoplasms or non-neoplastic lesions. Additionally, JCV DNA was widely present in different histological types of CNS neoplasms. With the exception of meningiomas, JCV Tag expression of CNS tumors was found in 36.7% (11/30) including 43.7% (7/16) astrocytomas, 22.2% (2/9) medulloblastomas /PNETs, and 40% (2/5) schwannomas. In contrast, only one (14.2%) of non-neoplastic CNS lesions expressed Tag protein. Therefore, JCV Tag expression was positively related to CNS neoplasms comparing with non-neoplastic lesions. Moreover, no JCV VP1 expressed in brain tumors and most non-neoplastic CNS lesions. The results suggest that JCV infects and potentially transforms cells in CNS.

INTRODUCTION

JC virus (JCV) is widespread in human population. Using serological studies, the prevalence of JCV infection is 70% of adult population [Padgett et al., 1973; Taguchi et al., 1982]. Primary infection occurs in childhood and persists latently within kidneys [Chester et al., 1983; Dorries et al., 1983; KiTamura et al., 1997]. In immunodeficient states such as autoimmune diseases, posttransplantation, malignancy or pregnancy [Arthur et al., 1988; Chang et al., 1996b, c; Coleman et al., 1980; Gardner et al., 1984; Myers et al., 1989; Tsai et al., 1997; Yogo et al., 1991], virus is reactivated or spreaded and shedded in infected urine. JCV genome encodes 2 early proteins (large tumor antigen, Tag, and small tumor antigen, tag) and 3 late structural proteins (VP1, VP2, VP3). Tag controls the viral replication and transformation. JCV lytically infects oligodendrocytes causing a fatal demyelinating disease termed progressive multifocal leukoencephalopathy (PML) [Padgett et al., 1971].

For non-lytic infection, earlier reports that JCV developed glioblastomas, medulloblastomas and other unclassified primitive tumors by inoculated hamsters [Walker et al., 1973; Zu Rhein et al., 1983; Zu Rhein et al., 1979]. Transgenic mice containing the early region of JCV expressed JCV Tag protein and generated primitive tumors mimicking human medulloblastomas [Krynska et al., 1999a]. Current study reported that JCV DNA and Tag protein could be detected in CNS neoplasms [Krynska et al., 1999b; Valle et al., 2001], colorectal cancers [Laghi et al., 1999] and coexpression of Tag and β-catenin in colon cancer cells [Enam et al., 2002]. The mechanism of tumorigenesis may be an interaction of JCV Tag and host cellular regulators related to pRb [Dyson et al., 1990; Helt et al., 2003] or P53-mediated [Kim et al., 2002] and Wnt signaling pathway [Gan et al., 2001]. The oncogenic potential of JCV in human is still undetermined. Therefore, we collected 36 CNS neoplasms and 7 non-neoplastic CNS lesions by using a nested polymerase chain reaction method for identifying viral DNA and immunohistochemistry for Tag viral protein expression to investigate the role of JCV infection associated with CNS neoplasms.

MATERALS AND METHODS

Human Brain Tissues

We collected 36 specimens of central nervous system (CNS) neoplasms acquired from the Department of Pathology at Chung Shun Medical University Hospital after surgical resection. These CNS neoplasms include 16 grade I to IV gliomas, 6 meningiomas, 9 medulloblastomas/ primitive neuroectodermal tumors (PNETs), and 5 schwannomas. Those gliomas included 6 low-graded and 10 high-graded astrocytomas. 7 non-neoplastic brain lesions were also submitted, containing 2 abscesses, 1 reactive gliosis and 3 arteriovenous malformations and 1 intracerebral hemorrhage. The histopathologic diagnosis and classification of the CNS neoplasms was made by Dr. H. Chang according to 1993 World Health Organization (WHO) classification [Kleihues et al., 1993].

Immunohistochemistry

Brain tissues were routinely fixed in 10% neutral buffered formalin for 16-20 hours followed by paraffin embedding. The sections with 4μm in thickness were used for histology and immunohistochemical analysis. Paraffin sections were stained with haematoxylin and eosin for histochemical analysis. The labeled streptavidin-biotin method, according to the manufacturer's instructions (Universal LSAB2 kit, DAKO), was performed for immunohistochemical study.

Immunohistochemical staining was employed with the primary antibodies including mouse monoclonal anti-SV40 large tumor antigen (Clone pAb416, 1:100 dilution, Oncogene Science), cross-reactive to JCV or BKV [Mann et al., 1984], and rabbit polyclonal anti-JCV VP1 protein (1:1600 dilution) [Chang et al., 1996a]. The 4μm paraffin sections were

deparaffinized in xylene and rehydrated through graded ethanol to distilled water. For antigen retrieval [Shi et al., 1991], paraffin sections were microwaved in a citrate buffer (pH 6.0) for 5 min, interval 1 min, and then microwaved for an additional 5 min. These sections were followed to cool for 20 min and washed by distilled water and then bathed in a TBS buffer. Finally, the sections were developed with DAB substrate, counterstained with haematoxylin and examined by light microscopy. SV40-transformed COS-7 cells were used as positive controls for anti-SV40 Tag. PML brain section was used as positive controls for ant-JCV VP1 (Data not shown). Normal mouse or rabbit serum instead of primary antibodies was used as negative controls.

Viral DNA Analysis by Nested Polymerase Chain Reaction (nPCR) and DNA Sequencing

Viral DNA was extracted from sections of paraffin-embedded brain tissues by using QIAGEN TM DNeasy tissue kit. 2 slices of 10μm thick tissues were deparaffinized and rehydrated. The DNA-containing fractions were digested with proteinase K (500μg/ml) for 12 to 18 h in a buffer containing 100 mM Tris-HCl and 10 mM EDTA (pH 8.0), and followed by phenol/chloroform extraction and ethanol precipitation. The concentration of total DNA was quantified by spectrophotometry (OD 260/280). The genomic DNA were stored at -20℃until used.

DNA samples were performed in an nPCR with two pairs of primers flanking the regulatory region of Human polyomavirus. The external primers were JBR1 (5'-CCTCCACGCCCTTACTTCTGAG- 3'; nucleotides from –45 to –21) and JBR2 (5'-GTGACAGCTGGCGAAGAACCATGGC- 3'; nucleotides from 265 to 289) [Chang et al., 1996d]. The internal primers were JBRNS (5'- GAGGCGGCCTCGGCCTC–3'; nucleotides from –6 to 11) and JBRNAS (5'-ACATGTTTTGCGAGCC–3'; nucleotides from 222 to 237). Each PCR reaction was performed in a total volume of 50μl with 1.25 unit of *EX Taq* DNA polymerase (TaKaRa). The initial step of PCR was 95℃ 4 min and cycles of denaturation at 95℃ 45 sec, annealing at 55℃ 1 min and elongation at 72℃ 1 min. The final extension at 72℃ 4 min was made for termination. There were 0.5μg DNA for the first PCR and 1μl of the first PCR product for secondary PCR. Both PCRs were performed for 40 cycles. Negative controls consisted of samples amplified in absence of template DNA. JCV-contained plasmid was used as positive controls. The nPCR products were ligated into a pGEM-T Easy Vector (Promega) and transformed into *E. coli*. The plasmid DNA carrying nPCR fragment was extracted from the *E. coli*. The inserted DNA was determined by DNA sequencing using ABI autosequencer. The company provided the sequencing protocol.

RESULTS

Of clinical specimens of 36 brain tumors, the detailed data including immunohistochemistry and molecular examination were described in TABLE I.
Of 16 grade I to IV gliomas, only 11 cases were performed by nested PCR for viral identification because of limited specimens. 18 out of 36 primary brain neoplasms contained the Human polyomavirus JCV DNA. Among the JCV-contained primary brain neoplasms, the tumor types included astrocytoma, medulloblastoma/ primitive neuroectodermal tumor (PNET), schwannoma and meningioma. The viral genotypes exhibited 12 JCV TW-1 and 6 JCV CY. The data showed that the presence of JCV was more common than BKV in primary brain neoplasms. In addition, JCV was present in different histological types of brain neoplasms.

Immunohistochemically, the Tag expression of tumors was found in 43.7% (7/16) astrocytomas, 22.2% (2/9) medulloblastomas /PNETs, and 40% (2/5) schwannomas. Tag and VP1 proteins both showed the nuclear immnoreactivity. The positive immunoreactive cases were defined by intensity and quantity of the stained cells. The positive-stained intensity was

more than acellular stroma and the quantity were more than 10% of positive-stained cells. The positive-stained cells of all positive immunoreactive cases were ranged from 10% to 70%. Low-grade astrocytomas were characterized by neoplastic astrocytes with mild pleomorphic nuclei and diffuse infiltration of the white matter (Fig. A, B). High-grade astrocytoma revealed nuclear marked atypia, frequent mitoses and vascular proliferation (Fig. C). Gliosarcoma diagnosed was composed of neoplastic astrocytes surrounded by sarcomatous cells. The Tag expressed in glial component (Fig. D). Primitive, undifferentiated medulloblastoma cells with round to oval nuclei contained focal Tag expression (Fig. E). Area with compact elongated neoplastic Schwann cells was shown (Fig. F). Negative Tag immunostaining was identified in meningioma characterized by mass growth of neoplastic meningothelial cells (Fig. G). COS-7 cells exhibited positive nuclear immunoreaction as positive controls (Fig. H). The exemplified microphotographs were listed in Figure.

None expressed Tag compared with a high JCV DNA detection rate (83.3%) in meningiomas. In total, viral JCV DNA sequence was identified in 18/31 (58%) and JCV Tag was expressed in 11/36 (30.5%) tested brain tumors. There was no evidence of VP1 protein expression in brain tumors. TABLE II recorded the presence of Human polyomavirus in 7 non-neoplastic brain lesions. Viral genotyping analysis showed JCV CY and JCV TW-1 types that were endemic in Taiwan [Chang et al., 2002]. Viral DNA of 7 JCV and 1 BKV were demonstrated. There were 1 case with coexisting presence of JCV and BKV DNA. Only one case (code no. 46) expressed Tag and structural VP1 proteins but no PML pathologic change in the sections examined. The remainders of non-neoplastic cases were no evidence of viral protein expression. In contrast to Tag expression of primary brain neoplasms, nearly none of Tag expression was noted in non-neoplastic brain lesions (TABLE III).

DISCUSSION

JCV was initially considered to replicate only in oligodendrocytes resulting in cell lysis and PML developed in human [Padgett et al., 1971]. In our study, non-neoplastic brain lesions all contained JCV DNA sequences. Additionally, we found JCV DNA sequences were detected in meningiomas, astrocytomas, medulloblastomas and schwannomas. Of these types of CNS neoplasms, meningiomas and schwannomas were firstly examined to detect the presence of the JCV DNA sequences. Therefore, the findings provide the evidence that brain is considered to be one of the sites of JCV latency, and JCV infects the different histogenic cells including meningothelial cell, glial cell, neural cell and Schwann cell. The results also confer that JCV infects cells not limited to oligodendrocytes, and support the tendency of JCV infection with wider tropism in CNS.

JCV may trigger cells to neoplastic change has been proved in animal studies [Review in Gary et al., 1998; Zu Rhein et al., 1979; 1983]. However, the mechanisms on dependence of viral Tag had been studied. In transgenic mice containing JCV viral early genome, undifferentiated primitive tumor was developed in mesentery or brain while higher-level production of JCV Tag protein in the tumor tissue but not in the non-tumor tissue [Franks et al., 1996; Krynska et al., 1999a]. Tag interacts with p53 protein [Staib et al., 1996; Kim et al., 2002] or pRB family molecules [Dyson et al., 1990], and subsequently, regulates viral and cellular genes involving tumor growth [Helt et al., 2003]. Recently, an alternative pathway was reported. JCV Tag via Wnt signaling pathway includingβ-catenin involved in transgenic mice developing cerebellar primitive neuroectodermal tumor. Western blot showed higher levels ofβ-catenin in JCV Tag-positive tumor cells than those in Tag-negative cells [Gan et al., 2001]. Therefore, Tag in the JCV-infected cells exhibits transforming ability.

To JCV and human CNS neoplasms, JCV DNA sequence had been detected in ependymomas, subependymomas, oligodendroctomas, medulloblastomas/PNET [krynska et al., 1999b; Valle et al., 2001] and glial-derived tumors [Boldorini et al., 1998;

Calsarelli-Stefane et al., 2000]. Additionally, earlier reports described that expression of JCV Tag in tumor cells in 28 (32.9%) of 85 tested brain tumors by using immunohistochemistry. Histological types of brain tumors with Tag expression consisted of oligodendrogliomas, astrocytomas, ependymomas and subenpendymomas [Valle et al., 2001]. Human pediatric medulloblastomas had been identified to contain 87% JCV DNA sequences and 25% JCV Tag protein expression by using PCR and immunostaining methods [Krynska et al., 1999b]. Thus, JCV Tag-mediated tumorigenesis was conferred to be one of the oncogenic mechanisms in CNS neoplasms. Our present study showed that positive detection rates of JCV DNA sequences was 58% (18/31) and Tag protein expression was 36.7% (11/30) in CNS neoplasms including astrocytomas, medulloblastomas and schwannomas. In non-neoplastic brain lesions, only one case (14.2%) showed the Tag protein expression. Additionally, JCV VP1 protein could not be detected in any neoplastic CNS neoplasms. We further implicated that JCV Tag expression seemed to be positively related to CNS neoplasms. The evidence of human CNS neoplasms with JCV Tag protein expression suggests to be linked to human CNS oncogenesis that JCV reactivated may trigger Tag-mediated pathway resulting in tumor growth.

Some studied samples exhibited JCV Tag protein expression but lacked any detectable JCV molecules. The “hit-and-run” hypothesis could be used to claim the situation that viral oncogene could mediate cellular transformation and subsequently, it might be silent or be loss of viral genomic molecules [Skinner, 1976]. The facts were recently demonstrated that adenovirus oncogenes, E1A and E1B genes, transformed primary rat kidney cells. The majority of transformed cells contained E1A proteins and lose E1A-specific DNA molecules [Nevel et al., 2001]. However, more studies need to clarify the oncogenic potential of JCV and human CNS neoplasms.

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Legend

Fig. Expression of JC virus large tumor antigen in various central nervous system neoplasms. A: Grade I astrocytoma, 400x. B: Grade II astrocytoma, 400x. C: Glioblastoma, 400x. D: Gliosarcoma, 400x. E: Medulloblastoma/PNET, 400x. F: Schwannoma, 400x. G: Meningioma, 400x. H. COS-7 cell, 200x. Brown color was developed by a diaminobenzidine substrate and blue color was counterstained with haematoxylin. 10 % to 50% of positive nuclear immunostained cells were present in A to F. Negative in G. Positive control in H.

TABLE I. Results of human polyomavirus in brain tumors by immunohistochemistry and nested polymerase chain reaction

Code no.	Age/ Gender	Histology	Immunohistochemistry		nPCR	Viral type
			Anti-LT	Anti-VP1		
3	11/F	Astrocytoma	+	-	+	JCV TW-1
4	11/F	Astrocytoma	-	-	ND	
14	70/F	Astrocytoma	-	-	-	
15	32/M	Astrocytoma	-	-	+	JCV TW-1
16	36/M	Astrocytoma	-	-	-	
8	60/M	Anaplastic astrocytoma	-	-	ND	
9	31/M	Anaplastic astrocytoma	-	-	ND	
5	61/F	Glioblastoma	-	-	ND	
6	35/F	Glioblastoma	+	-	+	JCV TW-1
7	63/M	Glioblastoma	+	-	-	
10	53/F	Glioblastoma	-	-	ND	
11	26/M	Glioblastoma	-	-	-	
12	59/M	Glioblastoma	+	-	+	JCV TW-1
13	47/F	Glioblastoma	+	-	+	JCV TW-1
38	41/F	Gliosarcoma	+	-	-	
30	27/M	Ganglioglioma	+	-	+	JCV CY
17	79/M	Meningioma	-	-	-	
19	55/F	Meningioma	-	-	+	JCV CY
20	61/M	Meningioma	-	-	+	JCV TW-1
21	81/F	Meningioma	-	-	+	JCV TW-1
22	48/F	Meningioma	-	-	+	JCV TW-1

65	65/M	Meningioma	-	-	+	JCV CY
24	7/M	Medulloblastoma/PNET	-	-	+	JCV TW-1
25	7/M	Medulloblastoma/PNET	-	-	+	JCV CY
27	7/M	Medulloblastoma/PNET	-	-	-	
29	2/F	Medulloblastoma/PNET	+	-	+	JCV CY
52	6/M	Medulloblastoma/PNET	-	-	-	
66	3/F	Medulloblastoma/PNET	-	-	-	
23	2/M	Medulloblastoma/PNET	-	-	-	
26	2/M	Medulloblastoma/PNET	-	-	+	JCV TW-1
50	3/F	Medulloblastoma/PNET	+	-	+	JCV CY
31	39/F	Schwannoma	-	-	-	
32	46/F	Schwannoma	-	-	-	
33	46/F	Schwannoma	+	-	+	JCV TW-1
34	30/F	Schwannoma	-	-	+	JCV TW-1
35	25/F	Schwannoma	+	-	-	

LT, large tumor antigen; VP1, structural VP1protein; nPCR, nested polymerase chain reaction

TABLE II. Presence of human polyomavirus in non-neoplastic brain lesions

Code no.	Histology	Immunohistochemistry		nPCR	Viral type
		Anti-LT	Anti-VP1		
51	Abscess	-	-	+	JCV CY
68	Abscess	-	-	+	JCV CY
44	AVM	-	-	+	JCV CY
48	AVM	-	-	+	JCV TW-1/BKV
49	AVM	-	-	+	JCV CY
46	Gliosis	+	+	+	JCV CY
43	ICH	-	-	+	JCV CY

AVM, arteriovenous malformation; ICH, intracerebral hemorrhage; LT, large tumor antigen; VP1, structural VP1 protein; nPCR, nested polymerase chain reaction; ＋, positive immunoreactivity; －, negative immunoreactivity

TABLE III. Summary of human polyomavirus in brain tumors or non-neoplastic lesions by immunohistochemistry and nested polymerase chain reaction

Brain Pathology	Immunohistochemistry			nPCR		Viral type	
	n	Anti-LT	Anti-VP1	n	Positive	JCV	BKV
Brain neoplasms							
Astrocytoma (G1-G4)	16	7	0	11	6	6	0
PNET/Medulloblastoma	9	2	0	9	5	5	0
Schwannoma	5	2	0	5	2	2	0
Meningioma	6	0	0	6	5	5	0
Total	36	11	0	31	18	18	0
Non-neoplastic lesions							
Abscess	2	0	0	2	2	2	0
AVM	3	0	0	3	3	3	1
Gliosis	1	1	1	1	1	1	0
ICH	1	0	0	1	1	1	0
Total	7	0	0	7	7	7	1

Positive case number in cells

LT, large tumor antigen; VP1, structural VP1 protein; nPCR, nested polymerase chain reaction