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Investigation of JCV-associated carcinogenesis in lung cancers

探討人類多瘤性病毒對肺癌致癌性之研究（93-2320-B-040-046-）

Abstract

JCV is a causative agent of progressive multifocal leukoencephalopathy. In addition to the brain, JCV reported has been limited to present in the extraneural organs. As with studying the relation of JCV and brain tumors, we found that the detection rate (43%) of JCV DNA sequences was higher in brain tissues of metastatic lung carcinomas than that of normal brain tissue. The aim of our study was in the highlight of the interaction between JCV and primary lung cancers. By using nested polymerase chain reaction, we found JCV DNA sequences could be identified in 57% (8/14) studied samples, 3 were squamous cell carcinomas and 5 were adenocarcinomas. Nucleotide sequence analysis showed archetype, Mad1 and their variants of JCV. To better understand characteristics of JCV infection, the immunohistochemical stains of anti-large tumor antigen (Tag) and anti-structural VP1 protein was performed in human lungs. JCV Tag could express in bronchial epithelial cells and lung cancer cells. There was no evidence of Tag protein expression in lymphocytes and pneumocytes of the lungs. Viral structural VP1 protein expression was not found in studied samples. We suggested that JCV could infect the lung tissue that might be a potential latent site.

KEY WORDS: JC virus; BK virus; lung

Introduction

JC virus, a member of the *Polyomaviridae*, is a double-stranded DNA virus containing early and late coding sequences by the viral regulatory region. Early proteins consist of large tumor antigen (Tag) expressing oncogenic potential in human and late proteins are structural proteins such as VP1 (Frisque *et al.*, 1984; Cole *et al.*, 1996). Epidemiological studies showed that JCV infection is widespread in human population. The serological surveys revealed that the JCV-specific serum antibody with peak seroconversion is up to 80% in adulthood (Brown *et al.*, 1975; Chesters *et al.*, 1983; Dorries *et al.*, 1997; Chang *et al.*, 2002; Padgett and Walker, 1973; Taquchi *et al.*, 1982; Walker *et al.,* 1986). JCV infection occurring at childhood is usually asymptomatic. After primary infection, the virus can cause a subacute, fatal, demyelinating brain disease called progressive multifocal leukoencephalopathy (PML), particularly in AIDS patients, and be excreted to the urine by unknown reactivated mechanism. Thus, the kidney and brain are thought to be the major latent sites of JCV (Dorris *et al.*, 1997; Jung *et al.*, 1975; Tominaga *et al.*, 1992; Yogo *et al.*, 1990).

To clinical investigation, JCV is initially considered to replicate with the limitation to the diseased brain tissue of PML (Padgett *et al.*, 1971). In addition to brain, JCV DNA sequences can be also detected in kidney, lymph node, heart and lung tissue of PML patients (Newman and Frisque, 1997). In non-PML individuals, JCV DNA has been isolated from several organs, including brain, lymphoid cells, tonsils, colon and prostate (Elsner *et al.*, 1992; Laghi *et al.*, 1999; Monaco *et al.*, 1998;Tornatore *et al.*, 1992; Zambrano *et al.*, 2002). The evidence seems to indicate that JCV can infect human cells with a board spectrum, not in restriction to those of PML patients. The oncogenic ability of JCV is demonstrated in animal studies (Franks *et al.,* 1996; Krynska *et al.,* 1999a; Walker *et al.,* 1973; Zu Rhein *et l.,* 1979; 1983), while there is no strong evidence between JCV and human cancers. Although the JCV has been reported in various human tumor tissues including brain tumors and colon cancers (Krynska *et al.*, 1999b; Laghi *et al.*, 1999; Ricciardiello *et al.*, 1998; Valle *et al.*, 2001), the oncogenic potential in human remains to be further studied.

The route of JCV transmission is still not clearly defined partly because the infected person is asymptomatic during primary infection. Detection JCV DNA in human tonsils provided the evidence for the initial site of JCV infection and possible transmission through the respiratory system (Monaco *et al.*, 1998). Recently, JCV DNA sequences and Tag expression were found in fetal lungs in our laboratory (unpublished data). In brain tissues metastasized by lung cancers, we also observed the presence of JCV DNA sequences. Therefore, we collected 17 surgical lung specimens of the patients with primary lung carcinomas to study the presence of JCV infection in human lungs.

Results

JCV DNA in brain tissue metastasized by the lung carcinomas

By using nested polymerase chain reaction (nPCR) with 2 sets of the primers specific to polyomavirus, we found 3 polyomavirus-positive cases in 7 studied samples with metastatic lung carcinomas to the brain (Table 1). Of 3 cases with polyomavirus infection, 2 were metastatic squamous cell carcinoma and 1 metastatic small cell carcinoma. Nucleotide sequencing revealed 3 cases with JCV infection and one case with a dual infection of JCV and BK virus (BKV). DNA sequencing of BKV was archetype, WW (Rubinstein *et al.*, 1987). JCV showed archetypes, CY (Yogo *et al.*, 1990) and Taiwan-1 (TW1) strains (Chang *et al.*, 1996). CY archetype contained three copies of GGGAA pentanucleotide at nucleotide 30-34, 198-202, and 218-222, and two copies of AAAGC pentanucleotide at nucleotide 188-192 and 207-211. With comparison to the regulatory region of the JCV CY, TW1 had a copy deletion of GGGAA pentanucleotide at 218-222. Higher detection rate (43%) of JCV infection in studied brain tissue was noted in comparison with 20% in normal brain tissue (Elsner *et al.*, 1992).

Association of JCV infection with primary lung cancers

To better understand whether JCV DNA present in metastatic lung carcinoma cells or in brain tissue, we collected 17 primary lung cancer tissues for further study. Those patients' profiles were summarized in Table 2. There were 13 male and 4 female. Their ages were ranged from 33 years to 88 years with an average of 67.8 years. There was no clinical evidence of PML and human immunodeficiency virus (HIV) infection in those patients at the diagnosis of primary lung cancers. Neither chemotherapy nor radiation therapy was treated before surgical resection of the lungs. Pathology of those lung cancers was classified into 11 squamous cell carcinomas and 6 adenocarcinomas.

By using the nPCR method, JCV regulatory region was sequenced from –6 to 237. JCV DNA sequences were isolated from 8 of 14 (57%) studied samples. There was no detection of BKV DNA. Of 8 JCV-positive samples, 3 were squamous cell carcinomas and 5 adenocarcinomas. The detection rate of JCV DNA was significantly higher in adenocarcinomas (100%) than in squamous cell carcinoma (33%) ($p<0.05$, Fisher exact test). There was no statistic significance between JCV detection and age or gender ($p>0.05$, Fisher exact test).

Nucleotide sequence analysis of JCV exhibited CY, TW3 and Mad1 as well as their variants. Samples L40 and L45 showed the sequences similar to the JCV CY besides point alterations (Figure 1A). L40 had 5 point mutations at nucleotide 21 (T→G), 34 (A→G), 37 (T→C), 169 (T→C) and 214 (C→A). One point alteration at nucleotide 110 (C→T) occurred in sample L45. Taiwan-3 (TW3) had two small deletion regions containing 5 nucleotides in each, located at nucleotide 188-192 and 218-222 of JCV archetype. Samples L39 and L50 contained JCV TW3 DNA sequences. However, samples L39 and L43 were variants of JCV TW3 (Figure 1B) in which each had one point mutation at nucleotide 9 (A→G) in L39, and nucleotide 20 (A→G) in L43. The regulatory region of JCV Mad1, the etiological agent of

PML, was characterized by two 98-bp repeats that feature a TATA box and by the absence of the 23- and 66-bp fragments that were present in the CY strain (Padgett *et al.*, 1976). The DNA sequence of JCV Mad1 was sequenced from nucleotide –6 to 246. Sample L44 demonstrated a JCV Mad1 genotype. Sample L41, Mad1-like, had 2 point mutations at nucleotide 34 (A→G) and 149 (A→G). We found that genoheterogeneity of JCV including CY, TW3 and Mad1 strains as well as their variants were present in lung cancer tissues.

As with the presence of JCV DNA sequences, we further examined the production of the viral early (Tag) and late (VP1) proteins by an immunohistochemical stain. We examined where the proteins expressed in tumor cells, lymphocytes, bronchiolar epithelial cells or pneumocytes of lung cancer tissues (Table 3). We found Tag protein expression in 7 out of 17 (41%) lung tumors including 4 squamous cell carcinomas and 3 adenocarcinomas. 3 lung tissues showed Tag protein expression in the bronchiolar epithelial cells (Figure 2). There was no evidence of Tag protein expressing in lymphocytes and pneumocytes. Moreover, no any immunolabeling of anti-VP1 could be identified in lung cancer tissues. The results suggest that JCV can infect bronchiolar epithelial cells and tumor cells. JCV in infected-cells can be reactivated and expressed Tag protein. Cells in human lung may carry JCV DNA as a potential latent site.

Discussion

This study showed the presence of JCV DNA sequences in the lung cancer tissue. Although JCV DNA sequences have been detected in various cancer tissues including brain tumors, colon cancers, and prostatic cancers (Laghi *et al.,* 1999; Valle *et al.,* 2001; Zambrano *et al.,* 2002), lung cancer tissue is first reported to harbor JCV DNA sequences. JCV DNA had been detected in the lung tissues from the PML patients (Newman and Frisque, 1997; Caldarelli-Stefano *et al.*, 1999). In addition to PML patients, there was no positive detection of JCV DNA in lung tissue from immunocompromised or non-immunocompromised individuals (Schatzl *et al.*, 1994; Caldarelli-Stefano *et al.*, 1999). Based on our study, the prevalent rate of JCV DNA in lung cancer tissue was 57%. Although we used the genomic DNA extracted from lung cancer tissues composed of primary lung cancer cells and non-neoplastic lung tissue to perform the nested polymerase chain reaction (PCR), the source of cells carrying JCV DNA could not be localized. However, the evidence supports the presence of JCV in lungs.

In our study, it is a novel event that JCV DNA sequences isolate from the lung tissue and Tag protein expresses in lung carcinoma cells and bronchiolar epithelial cells. The observation indicates that JCV can infect bronchiolar and lung carcinoma cells. Since JCV infection is highly prevalent in human by viral seroepidemiology (Padgett *et al.,* 1973; Brown *et al.,* 1975; Taquchi *et al.,* 1982; Chesters *et al.,* 1983; Dorries *et al.,* 1997; Chang *et al.,* 2002; Stolt *et al.,* 2003), respiratory inhalation is a common and possible route of transmission. In PML patients, JCV causes brain lesions are multiple foci in the white matter, so hematogenous spreading of viral particles has been suggested (Astrom *et al.,* 1958).

JCV infects tonsil stromal cells that are thought to the initial site, and tonsil lymphocytes serve as a reservoir for viral latency and reactivation (Monaco *et al*, 1996; Wei *et al*, 2000). More of the recent studies have focused on lymphocyte tropism of JCV, lymphocytes are introduced to the traffic of JCV from the initial infection site to the body organs including brain (Dorries *et al.,* 2003; Gallia *et al*, 1997; Sabath and Major, 2002). Our finding provides the evidence of the possibility of respiratory transmission of JCV particles.

JCV genome consists of non-coding regulatory regions, also called transcriptional control region (TCR). Based on the structure of TCR, JCV is divided into archetype and tandem repeat variants (Jensen *et al.*, 2001). The archetype (CY) that is originally isolated from the urine of the healthy individual (Yogo *et al.*, 1990) is presumed to be the circulating strain of JCV. In contrast to CY strain, tandem repeat variants are defined because of the deletions and duplications of viral sequences within their TCR (Jensen *et al.*, 2001). Of the tandem repeat variants, the Mad-1 is the etiology of human PML (Padgett *et al.,* 1976). Thus, tandem repeat variants are usually found in PML patients, in brain tumors and in lymphocytes of PML and non-PML people (Ault *et al.*, 1997; Calsarelli-Stefane *et al.*, 2000; Tornatore *et al.*, 1992). The presence of CY strain is related to the non-PML patients and rarely to the brain (Chang *et al.,* 1996a; 1996b; 2002). Taken together, the evidence provided that the specificity of the JCV strains could be decided in the sequence variations of TCR that might affect viral tissue tropism consequently.

In our previous study, we have found that JCV CY and TW1 (CY-like varient) strains were prevalent in Taiwan healthy individuals (Chang *et al.,* 2002), pregnant women (Chang *et al.,* 1996) and autoimmune disease patients (Chang *et al.,* 1996) by the PCR detection of JCV DNA in the urine. In contrast, sequencing analysis of these samples studied in this survey showed nucleotide diversity in JCV regulatory region. These brain tissues with metastatic lung carcinomas isolated JCV with CY or TW1 (CY-like) strain similar to the previous studies. No tandem repeat variants could be identified in the brain tissues. Alternately, primary lung cancer tissues harbored JCV DNA sequences composed of CY, TW3 (CY-like) and Mad1 strains. We found that tandem repeat strain, Mad1, was present in 2 primary lung cancer tissues with Tag expression. Accordingly, JCV Mad1 is first identified in Taiwan. Whether or not JCV Tag is related to the carcinogenesis of the lung, the event needs to be investigated.

Materials and methods

Tissue samples

A total of 24 clinical specimens were collected from the department of pathology at Chung Shun Medical University Hospital and China Medical University Hospital after surgical resection. Of those specimens, 7 samples were obtained from the patients having lung carcinomas with brain metastasis. Those patients were all male with the mean age of 59 years. They had neither the progressive multifocal leukoencephalopathy (PML) nor infected by human immunodeficiency virus. The remaining 17 samples were primary lung cancer tissues. The histopathologic diagnosis and classification of the lung cancers was made by Dr. H. Chang according to 1999 World Health Organization (WHO) classification (Travis *et al.,* 1999). Their medical records searched clinical data of those studied patients.

Immunohistochemistry

Brain and lung tissues were routinely fixed in 10% neutral buffered formalin for 16-20 hours followed by paraffin embedding. The sections with 4 μm in thickness were used for histology and immunohistochemical analysis. Paraffin sections were stained with haematoxylin and eosin for routine examination and pathological diagnosis. 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, specific BC loop (1:800 dilution). The 4 μm tissue sections were deparaffinized in xylene and rehydrated through graded ethanol to distilled water. For antigen retrieval, paraffin sections were autoclaved with 121℃, 15 lb in a citrate buffer (pH 6.0) for 20 min. These sections were followed to cool for 20 min at room temperature and washed by distilled water and then bathed in a TBS buffer. Finally, the brown color was developed with DAB substrate, counterstained with Gill's haematoxylin and examined by light microscopy. Positive immunoreactivity of anti-Tag or anti-VP1 exhibited a nuclear staining. SV40-transformed COS-7 cells and JCV-infected paraffin sections of fetal lungs (unpublished data) 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 formalin-fixed, paraffin-embedded studied tissues by using QIAGEN TM DNeasy tissue kit. To avoid the cross-contamination of the samples, we changed the microtome blade and cleaned the related experiments for each sample. 2 slices of 10 μm thick tissues were deparaffinized and rehydrated. The DNA-containing fractions were digested with proteinase K (500 μg/ml) for overnight 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). 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 50 μl 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. The templates contained 0.5 μg genomic DNA for the first PCR and 1 μl of the first PCR product for secondary PCR. Both PCRs were performed for 40 cycles. Contaminated 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.

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Table 1. The presence of JC virus in brain tissue of metastatic lung carcinomas

No.	Gender	Age (yr)	Organ	Pathology	Origin	hPy
B54	M	68	Brain	SQ. C. C.	Lung	JCV CY/ BKV
B55	M	65	Brain	S. C. C.	Lung	-
B56	M	65	Brain	S. C. C.	Lung	JCV TW1
B57	M	40	Brain	SQ. C. C.	Lung	JCV TW1
B58	M	40	Brain	SQ. C. C.	Lung	-
B59	M	68	Brain	SQ. C. C.	Lung	-
B60	M	68	Brain	SQ. C. C.	Lung	-

hPy, human polyomavirus; SQ. C. C., squamous cell carcinoma; S. C. C., small cell carcinoma

Table 2. Characteristics of patients and pathological results

No.	Gender	Age (yr)	Organ	Pathology	Stage	pTNM
L37	M	72	Lung	SQ. C. C.	IB	pT2N0M0
L39	M	41	Lung	SQ. C. C.	IV	pT4N2M1
L40	F	68	Lung	Adenoca.	IIIA	pT2N2M0
L41	F	33	Lung	Adenoca.	IIIA	pT2N2M0
L42	M	73	Lung	SQ. C. C.	IA	pT1N0M0
L43	M	68	Lung	Adenoca.	IB	pT2N0M0
L44	F	59	Lung	Adenoca.	IB	pT2N0M0
L45	M	77	Lung	Adenoca.	IB	pT2N0M0
L46	M	80	Lung	SQ. C. C.		
L47	M	80	Lung	SQ. C. C.		
L48	M	70	Lung	SQ. C. C.		
L49	M	80	Lung	SQ. C. C.		
L50	M	88	Lung	SQ. C. C.		
L51	M	59	Lung	SQ. C. C.		
L52	M	71	Lung	SQ. C. C.	IB	pT2N0M0
L53	F	69	Lung	Adenoca.	IIB	pT2N1M0
L54	M	65	Lung	SQ. C. C.		

SQ. C. C., squamous cell carcinoma; Adenoca., adenocarcinoma; M, male; F, female
Stage (1997 definitions): pT1, ≤3 cm; pT2, >3 cm; pT3, invasion of chest wall, diaphragm, pericardium and mediastinal pleura; pT4, invasion of mediastinum, heart, great vessels, carina, trachea, esophagus and vertebra; pN0, no regional lymph node metastasis; pN1, metastasis in ipsilateral peribronchial or hilar lymph node; pN2, metastasis in ipsilateral mediastinal or subcarinal lymph node; pN3, metastasis in contralateral mediastinal or hilar lymph node; pM0, no distant metastasis; pM1, distant metastasis

Table 3. Results of large tumor antigen (Tag) immunohistochemical and polymerase chain reaction (PCR) studies in primary lung cancer tissues

No.	Pathology	Tag immunohistochemistry				PCR
		Tumor	Lym.	Epi.	Pneu.	hPy
L37	SQ. C. C.	+	-	+	-	-
L39	SQ. C. C.	-	-	-	-	JCV TW3-like
L40	Adenoca.	-	-	-	-	JCV CY-like
L41	Adenoca.	++	-	+	-	JCV Mad1-like
L42	SQ. C. C.	-	-	-	-	-
L43	Adenoca.	-	-	-	-	JCV TW3-like
L44	Adenoca.	+	-	-	-	JCV Mad1-like
L45	Adenoca.	-	-	-	-	JCV CY-like
L46	SQ. C. C.	+	-	+	-	-
L47	SQ. C. C.	-	-	-	-	-
L48	SQ. C. C.	+	-	-	-	JCV TW3
L49	SQ. C. C.	-	-	-	-	-
L50	SQ. C. C.	+	-	-	-	JCV TW3
L51	SQ. C. C.	-	-	-	-	-
L52	SQ. C. C.	-	-	-	-	ND
L53	Adenoca.	++	-	-	-	ND
L54	SQ. C. C.	-	-	-	-	ND

hPy, human polyomavirus; SQ. C. C., squamous cell carcinoma; Adenoca., adenocarcinoma; Lym., lymphocytes; Epi., bronchiolar cells; Pneu., pneumocytes; +, positive-stained cells (grading: +, 0-25%; ++, 25-50%; +++, >50%); -, negative staining; ND, none done

Figure 1.

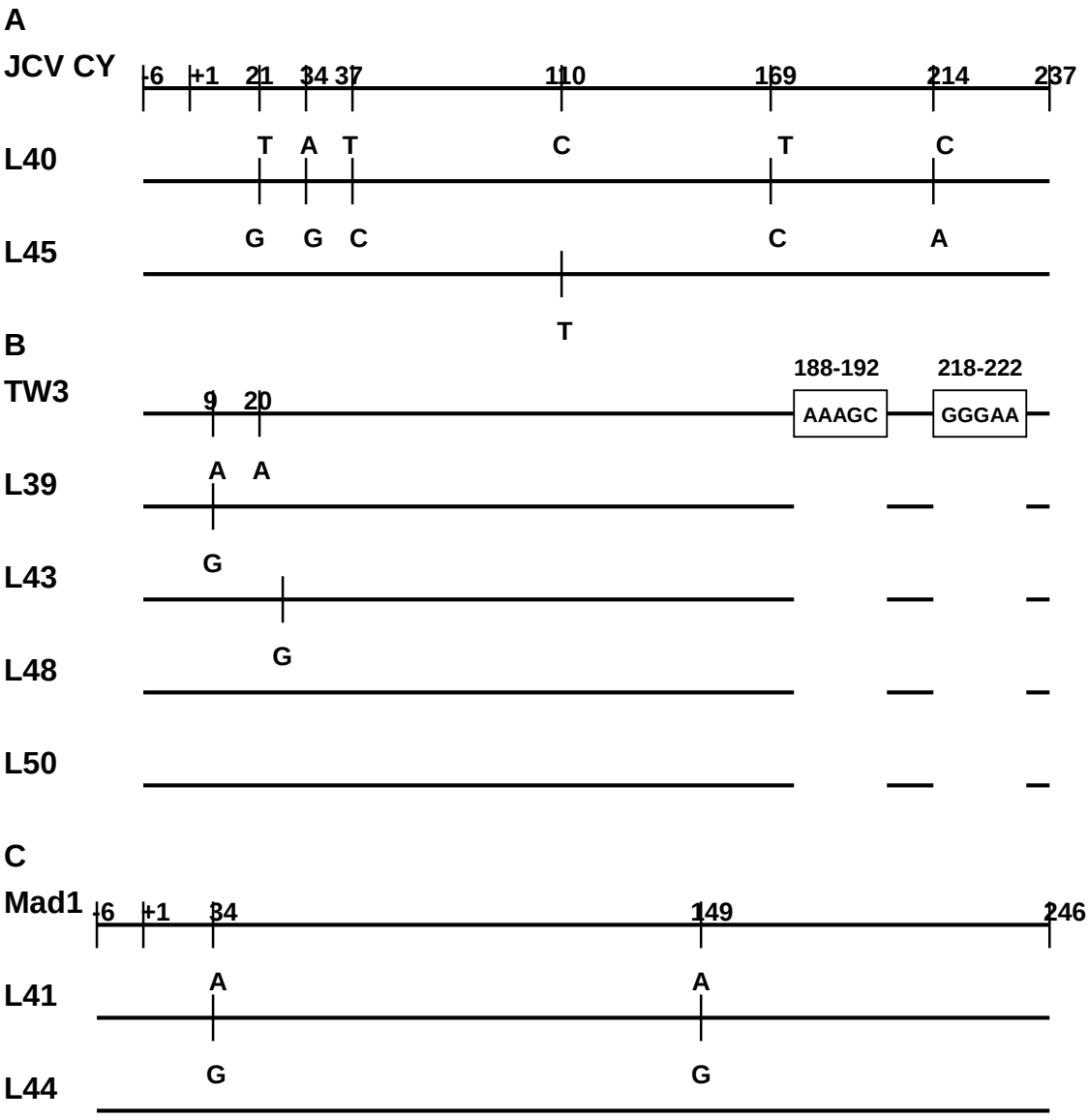

Figure 1. Nucleotide sequence diversity in JCV transcriptional control region from lung cancer tissues. The DNA sequences of samples L39, L40, L43, L45, L48, and L50 were sequenced from nucleotide –6 to 237. With comparison to JCV CY archetype (Yogo et al., 1990), TW3 (Chang et al., 1996) revealed two small deletion regions at nucleotide 188-192 and 218-222. Samples L48 and L50 were JCV TW3 genotype. The remainders were archetype-like variants (A, B). DNA sequencing of sample L44 was JCV Mad1 (Padgett et al., 1971) and L41 having two point mutations was classified as Mad1-like variant (C).

Figure 2.

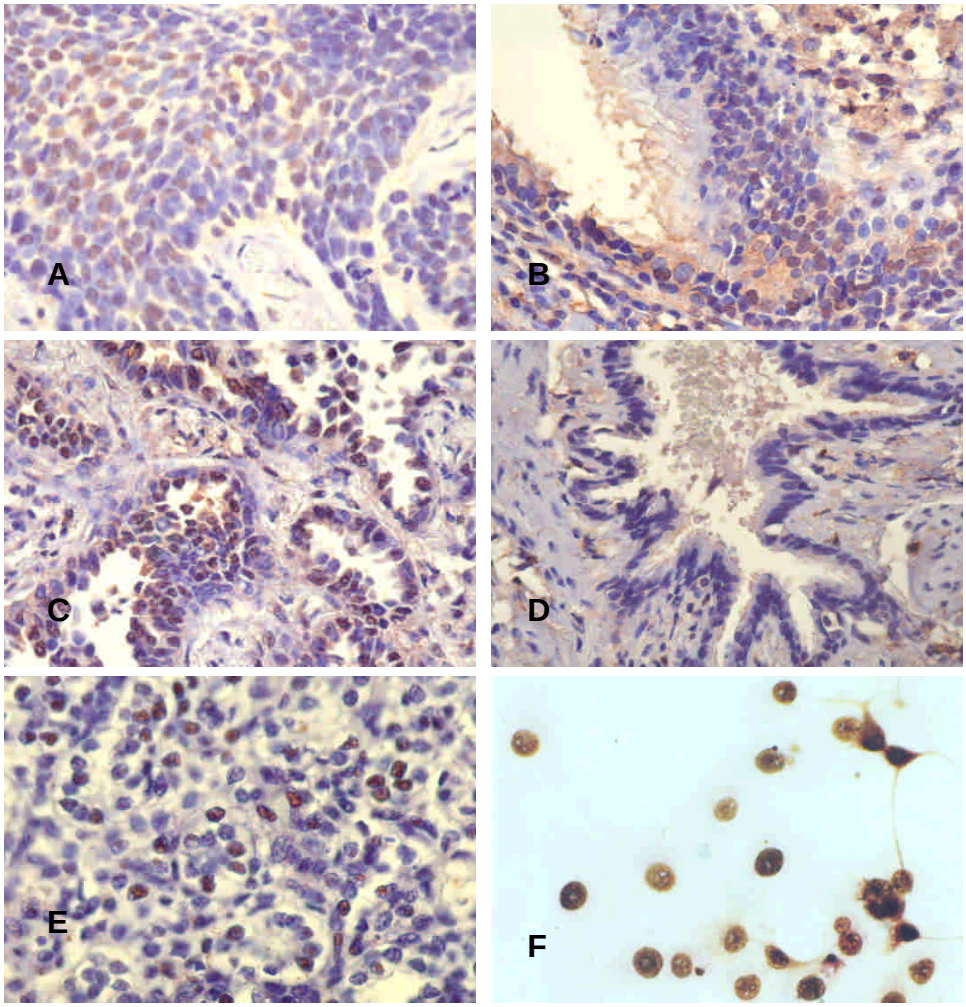

Figure 2. Anti-large tumor antigen (Tag) immunostaining in lung cancer tissues. A: Squamous cell carcinoma (sample L46) characterized by infiltrating islands of moderately differentiated, neoplastic squamous cells with nuclear pleomorphism. Positive nuclear immunostaining (brown color) was present in 40% of stained tumor cells. B: Bronchiolar epithelial cells adjacent to the tumor (sample L46) also showed 20% Tag-positive cells. C: Adenocarcinoma (sample L44) characterized by irregular neoplastic glands within the desmoplastic stroma. Tag-positive cells were 50% of stained tumor cells. D: Bronchiolar epithelial cells in the same section (sample L44) showed negative immunoreactivity. E: JCV-infected fetal lung paraffin section as positive control for anti-Tag. F: SV40-transformed COS-7 cells as positive control for anti-Tag.