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Detection of borna disease virus p24 RNA from human brain tissue in patients with central nervous system tumors in China

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【Abstract】 *Objective*: It intended to examine whether there is BDV infection in the human tumor tissues of central nervous system in China and investigate the correlation between BDV infection and tumors of central nervous system. *Methods*: Nested reverse transcriptase polymerase chain reaction(nRT-PCR)and fluorescence quantitative polymerase chain reaction(FQ-PCR)was used to detect the BDV p24 fragments in 60 samples of human tumor tissues of central nervous system and 14 normal brain tissues. *Results*: The study indicated the positive rate of the BDV p24 fragment in human tumor tissues of the central nervous system (6.67%)was higher than that in normal brain tissues(0), but no statistical significance($P>0.05$). *Conclusion*: It suggests that the BDV infection is present in the human tumor tissues of central nervous system in China, while the sample size wasn't large enough and we could not certify the possible correlation between BDV infection and cenfral nervous system tumors.

【Key words】 Borna disease virus; Central nervous system tumors
【Document code】 A

1 Introduction

Borna disease virus (BDV) is a species in the genus Bornavirus, family Bornaviridae, which has a nonsegmented, negative-sense, single-stranded ribonucleic acid(RNA) genome[1]. It is a noncytolytic neurotropic virus that infects a wide variety of animal species from birds to primates and causes Borna disease (BD), which is characterized by central nervous dysfunction with various manifestation ranging from fatal neuronal damage to almost asymptomatic viral persistence[2].

Previous researchers have detected BDV genome in the brain tissue of schizophrenic patients, but failed to detect BDV RNA in the peripheral blood monouclear cells(PBMCs) of the same person[1]. These results suggest that human brain tissues harbor the BDV genome even in the absence of BDV RNA in PBMCs of patients. The detection and isolation of BDV from human brain may be a key to confirm the role of BDV infection in human neuropsychiatric diseases.

BDV provides an important model for the investigation of the mechanisms and consequences of viral persistence in the central nervous system (CNS). Persistent infection with BDV has been established in a variety of neuronal and glial cell lines (e.g. astrocytes and oligodendrocytes)of human and animalsin in vitro experiments[3]. Also, BDV RNA was detectable *in CNS tumors(glioblastoma multiforme)from patients with immunosuppressive states*[4]. But little knowledge is available on whether there is BDV infection in human tumor tissues of CNS in China and the correlation between BDV infection and CNS tumors.

In our present study, we chose human tumor tissues of CNS and normal brain tissue of cerebral trauma as the samples which were detected by nRT-PCR and FQ-PCR. Here we documented, for the first time, the detection of BDV RNA from human brain tissue in a patient with glioma and 3 patients with meningioma in China.

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2 Materials and methods

2.1 Subjects

Brain tissue specimens from 60 patients with CNS tumor and 14 patients with cerebral trauma (table1) who resid-

ed in Chongqing and Zunyi (a city in Guizhou province)in the southwestern China were examined for BDV infection. All patients were inpatients from January 2002 to December 2003, in the departments of neurosurgery,the First Affiliated Hospitals of Chongqing Medical University or Zunyi Medical College. Informed consent was obtained from all the participants.

2.2 Tissue collection and preparation

CNS tumor tissue samples were collected from the operation,which were divided into two parts. One was stored in liquid nitrogen(LIN) at -196℃, the other was fixed in 10% formalin and sent to histopathological examination.

Normal brain tissue samples were collected from the internal decompression and stored in liquid nitrogen(LIN) at -196℃.

2.3 Preparation of RNA

Total cellular RNA was prepared from tumor tissues and normal brain tissues with an RNA extraction kit (Tripure; Roche Molecular Biochemicals, Indianapolis,USA)according to the manufacturer's manual. The approximate concentration of extracted RNA was determined by spectrophotometry. The extracted RNA was stored in 75% ethanol at -20℃.

2.4 Detection of BDV P24 fragments by nRT-PCR and FQ-PCR

In this study,BDV P24 fragment was detected by nRT-PCR and FQ-PCR. While routine nRT-PCR was performed,one specific oligonucleotide probe with 2 fluorescent groups was added in the total mixture for the second PCR.

Primer pairs used for the transcription and first PCR were 5'-TGACCCAACCAGTAGACCA-3'(P24-OF: nucleotides (nt) 1387-1405) and 5'-GTCCCATTCATCCGTTGTC-3'(P24-OR: nt 1865-1847). The second pairs of primer were 5'-CCCTC CAAGTGGAAACCAT-3'(P24-MF:nt 1609-1627) and 5'-CA GTATCTTGATGTTCTCGCCA-3'(P24-MR:nt 1694-1673). All primers were synthesized by BIOASIA biotechnology Co.,Ltd. The fluorescent probe were synthesized by Da An Gene CO.,LTD of Zhong Shan University, labeled with fluorescence 6-carbox y fluorescein(FAM)at the 5'end and tetramethylrho damine(TRAMA)at the 3'end as follows:5'-FAM- TCAG CGGTGCGACCACTCCGATAGC-TAMRA-3'(nt 1637-1661).

At first,reverse transcription were performed at 37℃ for 1 hour followed by 95℃ for 3 min (inac tivating reverse transcriptase) in a 10 μl(total volume) mixture containing approximate 1 ul of cellular RNA,2μl 5 × reverse transcription buffer (containing 25mmol/L $MgCl_2$),0.2 μl P24-OF and 0.2μl P24-OR(0.2 μg/μl),1 μl deoxy-ribonucleoside tr ipho sphate (10 mmol/L dNTP),1 μl MMLV (Moloney Murine Leukemia Virus) reverse transcriptase(20u), and 5.6 μl and diethypyrocarbonate(DEPC) treated water. Then the first PCR was performed in Gene Amp PCR system 9600 (Perkin Elmer,USA)in a 50 μl mixture containing 10 ul reverse transcription pro ducts,10 μl 5 × PCR buffer(containing 25 mmol/L $MgCl_2$),1μl P24-OF and 1μl P24-OR,1μl dNTP, 1μl Taq DNA polymerase (3u/μl),and 26 μl dei onized dis tilled water(ddH_2O). The condition of the first PCR was as follows:1 cycle at 94℃ for 2 min;40 cycles at 94℃ for 1 min,55℃ for 1 min,and 72℃ for 1 min; and 1 cycle at 72℃ for 7 min. Finally the second PCR was performed in ABI PRISM 7700 Sequence Detector (Perkin Elmer,USA) in a 50μl mixture containing 5μl products of the first PCR,10μl 5 × PCR buffer (containing 25 mmol/L $MgCl_2$),1μl P24-MF and 1μl P24-MR,1μl dNTP,1μl Taq DNA polymerase,1μl fluorescence labeling probe,and 30 μl ddH_2O. The condition of the second PCR was as follows:1 cycle at 93℃ for 2 min;10 cycles at 93℃ for 45s,55℃ for 1 min;and 30 cycle at 93℃ for 30s,55℃ for 45s.

RT-PCR for β-actin(internal reference) mRNA was car ried out by using total cellular RNA,to evaluate the quality of RNA extraction,and to avoid the false negative of BDV RNA. The nRT-PCR and FQ-PCR without the RT enzyme in the reverse transcription step were performed to be the negative control. The nRT-PCR and FQ-PCR was repeated in BDV RNA positive cases,and if a sample showe d positive twice,it could be regarded as positive. The positive PCR products were analyzed by 2% agarose gel electrophoresis.

2.5 Establishment of standard curve

For the quantitative detection of BDV in brain tissues, BDV recombinant plasmid cDNA was created and quantitated as standard template in our labor atory. Serial 10-fold dilutions of template were prepared ranging from 10^5 to 10^2 copies/μl,and PCR amplification was performed in ABI PRISM 7700 Sequence Detector according to the manufacture's instruction. Then the computer drew the standard curve automatically(Fig.1). The standard curve was constructed by plotting each of the thres hold cycle numbers(Ct values) against the log of the starting copy numbers for the standard template.

2.6 Prevention of contamination

To prevent possible contamination of the samples,four distinct areas were established,including a reagent preparation area,a specimen preparation area nested area, and a general work area. Tissue preparation,RNA extraction and cDNA amplification were carried out in separate rooms following strict rules. A separate set of micropipettes with plugged tips was used in the different areas. We also included a control sample(sterile water)to detect possible cross -contamination between the specimens.

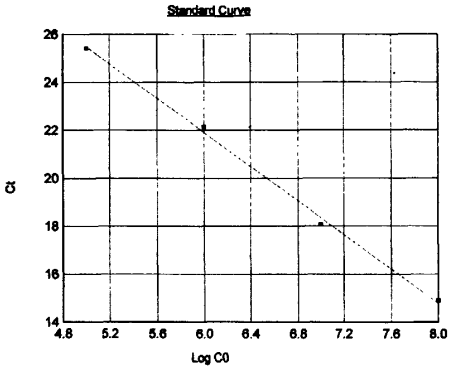


Fig.1. The standard curve of testing BDV p24 by FQ-PCR

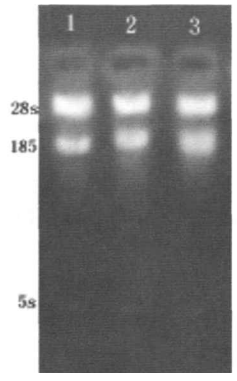


Fig.2. The results of RNA extraction

2.7 Statistical analysis

To examine the statistical significance of the differences between study groups, Fisher's exact probability method was performed with the assistance of commercially available software SAS6.12. Significance was placed at $P< 0.05$.

3 Results

3.1 The results of RNA extraction

The result of the 1% agarose gel electrophoresis indicated that the RNA extracted from tumor tissues and normal brain tissues had high purity and strong integrity(Fig.2).5 μl total RNA of CNS tumor tissues was diluted 100 times,then detected the OD value by ultraviolet spectrophotometer(Shimadzu Corporation). The OD_{260} was 0.474,OD_{280} was 0.251, OD_{260}/OD_{280} was 1.89,RNA concentration was 1.9μg/μl.

3.2 Detection of BDV P24 fragment in CNS tumor tissues and normal brain tissues

The RNA specimens were detected by nRT- PCR and FQ-PCR to evaluate the prevalence of BDV in CNS tumor tissues and normal brain tissues.

The results of BDV RNA in CNS tumor tissues and normal brain tissues identified by nRT -PCR and FQ-PCR indicated that 4 samples were positive(Fig.3).

The result of the positive PCR products analyzed by 2% agarose gel electrophoresis indicated that the RNA detected from tumor tissues was BDV p24 fragment(Fig.4).

According to the standard curve(Fig.1), the copy numbe rs of the 4 positive samples were calculated, which were $8.35 \times 103/\mu l$, $1.24 \times 102/\mu l$, $5.44 \times 103/\mu l$, $1.29 \times 102/\mu l$.

There were 4 positive of BDV p24 in tumor tissues in 60 patients with CNS tumor (4/60,6.67%),which contained 3 patients of meningioma (3/18,16.67%) and 1 patient of glioma (1/15,6.67%) (Table 2). While there was no positive in

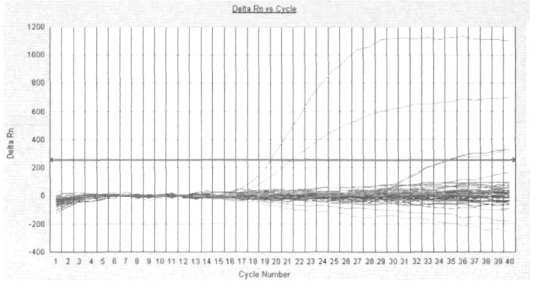


Fig.3. The curve of fluorescence delta Rn vs amplification cycle number of BDV p24 in CNS tumor tissues and normal brain tissues by nRT-PCR and FQ-PCR

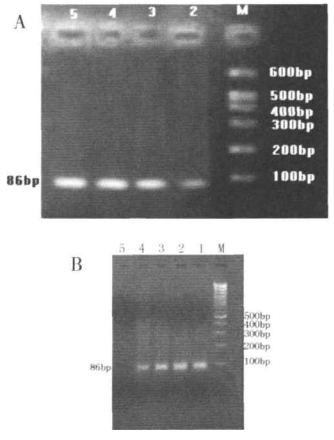


Fig.4. Electrophoretogram of BDV p24 fragment in standard template and human tumor tissues of central nervous system.

other tumor tissues or normal brain tissues.

The positive rate of BDV p24 in CNS tumor tissues (6.67%) was higher than that in normal brain tissues but without statistical significance(Fisher's exact probability method, $P>0.05$). The positive rate of BDV p24 in meningioma

(16.67%) was much higher than that in normal brain tissues, but without statistical significance either(Fisher's exact probability method, $P>0.05$), because the sample size wasn't large enough.

4 Discussion

BDV was named after the town of Borna in Saxony, Germany, where an epidemic of infectious e ncephalitis caused a large number of equine deaths in 1885[5]. In 1990, BDV was isolated from brain tiss ues of animal and determined to be a negative-sen se, single-stranded RNA virus[6]. The golden stan dard for diagnosing viral infections of the central nervous system has been the detection and isolation of virus from brain tissue. It is hard to obtain the brain tissue samples, so the researchers have to choose the body fluids such as peripheral blood and cerebrospinal fluid (CSF) instead, which are much easier to be obtain. More and more serological data and molecular epidemiological studies indicated that BDV could persistently infect the nervous system of human and might be associated with certain neuropsychiatric diseases[7-9]. Our previous studies have also fo und that there were BDV infections in China, which may be possibly associated with neuropsychiatric diseases[10-12]. Several studies have reported th e detection of BDV antigen and BDV RNA in bra in tissue collected at autopsy from patients with clinical records of mental disorders and healthy people [13,14], and the isolation of human-derived BDV(BDVHuP2br) in brain tissue collected at autopsy from a schizophrenic patient. Moreover, BDV RNA has also been detected in clinical samples of grade 4 glioblastomas. Consistent with these findings, we have detected BDV RNA in brain tissue of a glioma patient. At the same time, we detected BDV RNA from brain tissue in 3 patients with meningioma, which haven't been reported before. Our results showed evidence of BDV infection in the brain tissues of Chinese. We reported, for the first time, the detection of BDV RNA from brain tissues in patients with CNS tumors in China.

Some animal viruses may induce tumors in other species, which are non-tumorigenic in their natural hosts[15]. For example, herpesvirus saimiri an d herpesvirus ateles induce lymphoproliferative tumors[16], rhesus monkey polyomavirus SV40 present i n human mesotheliomas and brain tumors [17,18], orf virus and bovine papular stomatitis virus induce benign epitheliomas or occasionally histocytomas[19] and animal papillomavirus induce extensive verrucosis on butchers' hands [20]. Meanwhile, BDV is a noncytolytic, neurotropic RNA virus, BDV RNA was detected in patients with CNS tumors (glioblastoma multiforme) in Japan. These observations have led to the hypothesis that viruses can contribute to a variety of tumors. Therefore, there is much interest in identifying potential viral culprits.

The formation of CNS tumor is a complex course, which is correlated to oncogene activation and a ntioncogene inactivation. Now more and more attentions are paid to the relationship between virus infections and antioncogene inactivation.

Recent studies have suggested that BDV infection may cause direct damage in the developing brain by inhibiting the function of amphoterin(HMGB1) due to binding by the p24 phosphoprotein [21]. And p24 phosphoprotein directly binds to th e A-box domain on HMGB1, which is also responsible for interaction with a tumor suppression factor, p53[22]. Other studies have indicated that HMG B1 enhances p53-mediated transactivation of the promoter by direct binding to p53 via its A-box do main [23]. Mammalian two-hybrid analysis revealed th at p53 and p24 phosphoprotein competitively interfere with the binding of each protein to HMGB1 in a p53-deficient cell line, NCI-H1299 and p24 phosphoprotein was able to significantly decrease p53-mediated transcriptional activation of the cyclin G promoter[22]. Therefore, BDV p24 phospho protein may be a unique inhibitor of p53 activity via bin ding to HMGB1, which may be the possible mechanism of BDV-induced tumor.

In our study, the BDV p24 fragments were detected by nRT-PCR and FQ-PCR in human CNS tumor tissues. But the results had no statistical significance ($P>0.05$), because the sample size wasn't large enough. We had to admit that there was inadequate evidence to support BDV infection increased tumor incidence or a direct role for BDV in human cancer. In summary, our detection of BDV RNA in brain tissues of CNS tumor patients in China, showed direct evidence to support that BDV infection is present in the human tumor tissues of central nervous system in Chinese. And fur ther researches on BDV and CNS tumors are nee ded to make sure whether there is association between them.

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的生化标记物。有助于研究NSE在脑损伤中的作用机制，为判断中枢神经系统的损害程度提供了定量信息以及对预测病人出院后神经症状和体征的恢复程度提供了重要的参数，也可能有效地反映VE患者颅内压的高低，为临床合理使用降颅压药物，提供了新的监测手段。目前的研究多集中在脑损害后它们的动态改变以及与神经功能紊乱的程度和预后的相关性上，是否与脑的修复机制有关以及是否可以通过药物来改变它们的浓度等问题还有待进一步研究。

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