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Identification of the Novel KI Polyomavirus in Paranasal and Lung Tissues

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KI is a novel polyomavirus identified in the respiratory secretions of children with acute respiratory symptoms. Whether this reflects a causal role of the virus in the human respiratory disease remains to be established. To investigate the presence of KIV in the respiratory tissue, we examined 20 fresh lung cancer specimens and surrounding normal tissue along with one paranasal and one lung biopsy from two transplanted children. KIV-*VP1* gene was detected in 9/20 lung cancer patients and 2/2 transplanted patients. However, amplification of the sequence coding for the C-terminal part of the early region of KIV performed on the 11 positive cases was successful only in two malignant lung tissues, one surrounding normal tissue, and 1/2 biopsies tested. Phylogenetic analysis performed on the early region of KIV (including the four Italian isolates), BKV and JCV revealed the presence of three distinct clades. Within the KIV clade two sub-clades were observed. A sub-clade A containing the four Italian strains, and a sub-clade B comprising the Swedish and Australian isolates. Interestingly, the two Italian strains identified in normal tissue clustered together, whereas those detected in malignant tissue fell outside this cluster. In vitro studies are needed to investigate the transforming potential of KIV strains. ***J. Med. Virol. 81:558–561, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: KIV polyomavirus; human tumors; molecular epidemiology

INTRODUCTION

Polyomaviruses are small non-enveloped double-stranded DNA viruses that can infect mammals and birds. Until recently, two human polyomaviruses BKV [Gardner et al., 1971] and JCV [Padgett et al., 1971] were known to infect human beings. Both viruses are widespread in the human population, establish a latent infection in the kidney [Chesters et al., 1983], and can be detected in the urine of healthy individuals, where infection is asymptomatic [Brown et al., 1975].

In immunocompromised patients, reactivation of BKV and JCV may cause severe disease. JCV can cause a demyelinating disease known as progressive multiphocal leukoencephalopathy (PML) which occurs mainly in AIDS patients [Gordon and Khalili, 1998]. BKV can be responsible of a tubular nephritis, which can lead to allograft failure in renal transplant recipients and hemorrhagic cystitis in hematopoietic stem cell transplant recipients [Nickeleit et al., 2002; Hirsch and Steiger, 2003; Chen et al., 2004].

Polyomaviruses BKV, JCV, and SV40 have been detected in human tumors [Sariyer et al., 2004; Giuliani et al., 2007, 2008] and induce numerous tumors in experimental animals [Sariyer et al., 2004]. The mechanism by which the oncogenic transformation is achieved is through the action of the early proteins large T antigen (T-Ag) and small t antigen (t-Ag). The T-Ag binds the oncosuppressor proteins p53 and pRb, two key regulators of cell cycle progression [Bollag et al., 1989; Cress and Nevins, 1996] inhibiting apoptosis and driving the cell into S phase. The small t antigen interacting with the protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation [Sontag et al., 1993]. This aberrant stimulation of the cell cycle is responsible for oncogenic transformation.

Recently, a new human polyomavirus, KI polyomavirus (KIV) has been identified in the respiratory secretions of children with acute respiratory symptoms

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[Allander et al., 2007]. Debated is its causal role in the respiratory disease [Bialasiewicz et al., 2007; Norja et al., 2007; Babakir-Mina et al., 2008] as completely unknown is its oncogenic potential. The objective of the study was to determine the presence of KIV in normal and malignant lung tissue as well as in a normal paranasal tissue and to compare the early region of KIV genome, comprising the C-terminal region of the *T-Ag* gene and almost the entire *t-Ag* gene [Allander et al., 2007] with those of known KIV, JCV, and BKV strains.

MATERIALS AND METHODS

Study Group

A series of 20 fresh lung cancer specimens and surrounding normal tissue provided by the Department of Surgery of the Santo Spirito Hospital, Pescara, Italy, between 2005 and 2006 years were tested for the presence of the novel polyomavirus KI. These specimens were already tested for the presence of HPV, SV40, BKV, JCV, and CMV [Giuliani et al., 2007]. The patients included 13 men and 7 women and the age ranged from 40 to 85 years, mean age 68. In addition, one paranasal and one lung biopsy obtained from two thalassemic transplanted children (13 and 3 years old, respectively) were included in the study. Informed consent was obtained from all patients enrolled in the study.

DNA Extraction and Viral Detection

Twenty-five milligrams of normal and malignant tissue from each patient was digested overnight at 56°C with proteinase K and then total DNA extracted with the QIAamp DNA mini kit according to the manufacturer's instruction (Qiagen S.P.A., Milan, Italy). The DNA was eluted in a volume of 50 μl and stored at −80°C until analysis.

Amplification of the VP1 Gene and Early Region of KIV Genome

The KIV was detected amplifying a fragment of the *VP1* gene as described [Allander et al., 2007]. The positive cases were then amplified for the early region of KIV genome comprising the C-terminal part of *T-Ag* gene and the almost entire *t-Ag* gene [Allander et al., 2007]. The PCR primers were as follows: FT-Ag 5′-CCACACAGGTGGTTTTCTATAA-3′ (position 4521–4542) and RT-Ag 5′-GCCCCAGCTTTTTCTAGAAGAA-3′ (position 5032–5011). The cycling conditions were: 10 min at 94°C, 1 cycle; followed by 35 cycles of amplification at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. The PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. All the necessary precautions were taken to avoid contamination.

Sequencing of the Early Region and Phylogenetic Analysis

The amplified fragments of the early region of KIV were sequenced using the Genome Lab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) and run on a Beckman Coulter CEQ2000XL sequence analyzer after column purification. The obtained sequences were submitted to the Genebank and matched against all deposited sequences (http://www.ncbi.nlm.nih.gov/BLAST). An alignment with a set of reference sequences was obtained using CLUSTAL X [Thompson et al., 1994] and manually edited with the Bioedit software [Hall, 1999]. Positions containing gaps were removed from the final alignment. For our data set, the best fitting nucleotide substitution model was tested with a hierarchical likelihood ratio test following the strategy described by Swofford and Sullivan [2003], using a neighbor-joining (NJ) base-tree with LogDet corrected distances. Maximum likelihood (ML) trees were then inferred with the selected model and ML-estimated substitution parameters. The heuristic search for the ML tree was performed using an NJ tree as starting tree and the TBR branch-swapping algorithm. NJ trees were also estimated using pair-wise distances inferred by ML with the best fitting nucleotide substitution model. Calculations were performed with PAUP* 4.0b10 according to Swofford and Sullivan [2003]. Statistical support for internal branches in the NJ trees was obtained by bootstrapping (1,000 replicates) and with the ML-based zero-branch-length test for the ML trees [Swofford and Sullivan, 2003]. Trees were rooted by outgroup rooting using BKV-ETH-3 strain as outgroup. The accession numbers of the sequences utilized for the phylogenetic analysis including those referred to KIV strains identified in our laboratory are the following: AB263916; AB269841; AB269826; AB211387; AB269851; AB269822; AB211370; AY628225; AY628234; AY628232; AY628233; AY628231; AY628230; AY628229; AY628227; AY628226; AB127343; AB127018; AY536242; AB372037; AB113124; AY382184; AB038255; AB212954; AB212952; AF004349; AF030085; AY536239; EF520287; EF520288; EF520289; EF127906; EF127907; EF127908; FJ389513; FJ389514; FJ389515; FJ389516.

RESULTS

Detection of KIV-DNA

KIV-*VP1* gene was detected in 9 out of 20 lung cancer specimens tested (45%) and in 1 case the *VP1* gene was detected either in normal as well as in malignant tissue. Following this primary screening, a second PCR was performed on the nine positive cases targeting the early region of KIV. A positive result was obtained only in three cases: one surrounding normal tissue (KIV-RM1) and two malignant tissues (KIV-RM3 and KIV-RM4).

In the two transplanted thalassemic children, the *VP1* gene was detected in both cases while amplification of the early region of the viral genome was positive only in one case (KIV-RM2).

Phylogenetic Analysis

Phylogenetic analysis revealed the presence of three main clades, supported by highly significant bootstrap and *P* values in the zero-branch-length test. Two

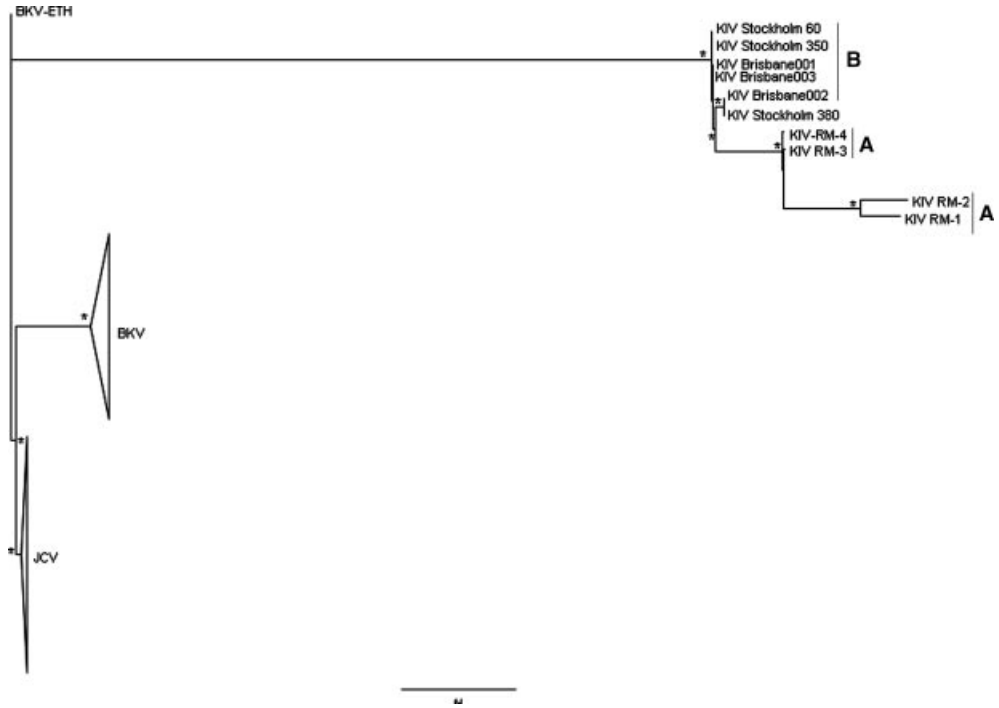

Fig. 1. Phlylogenetic analysis of the early region (512 nt) of human polyomaviruses was carried out using HKY + I + C (alpha-parameter = 0.0763) as the best evolutionary model. Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test [Posada and Crandall, 1998], and were drawn in scale with the bar at the bottom indicating 0.3 nucleotide substitutions per site. One * along a branch represents significant statistical support for the clade subtending that branch ($P < 0.001$ in the zero-branch-length test) and bootstrap support >75%. The tree was rooted using the strain BKV-ETH-3 as outgroup. Sub-clades within the main KIV clade are indicated with the letters A and B.

represented BK and JC polyomaviruses (Fig. 1) while the third one contained the novel KIV strains. This third clade can be subdivided into two statistically supported sub-clades, named A and B, containing the four Italian isolates and the Swedish and Australian isolates, respectively (Fig. 1; $P < 0.001$ in the zero-branch-length test, Bayesian posterior probability >90%, and bootstrap support >75%.). Within sub-clade A, KIV-RM1 and KIV-RM2 resulted more closely related compared to KIV-RM3 and KIV-RM4 isolates which were placed outside this cluster.

The mean genetic distance between groups (BKV, JCV, and KIV) was 0.26, whereas within clades A and B was 0.19 and 0.137, respectively. These values were not statistically significant. Conserved mutations/motifs were not observed in the virus infecting the neoplastic tissue.

DISCUSSION

The novel polyomavirus KIV was first described by Allander et al. [2007] in the respiratory secretions of children with acute respiratory symptoms and later confirmed by other groups around the world [Bialasiewicz et al., 2007; Babakir-Mina et al., 2008]. Whether this reflects a causal role of the virus in the respiratory illness is still debated and molecular epidemiological studies are needed to establish a firm link with the respiratory disease. Up to now, completely unexplored is the oncogenic potential of KIV.

The human polyomaviruses BKV and JCV have been detected in several human tumors [White and Khalili, 2004; Giuliani et al., 2008] including the respiratory tract [Giuliani et al., 2007; Zheng et al., 2007], and induce tumors in animal models [Sariyer et al., 2004]. This prompted us to investigate the presence of KIV in normal and malignant respiratory tissue. PCR analysis revealed the presence of KIV-*VP1* gene in some of the lung cancer tissues examined and in both normal biopsies (paranasal and lung) from the transplanted patients. However, when the same positive samples were tested for the presence of the early region of the viral genome, an amplified product was obtained only in four of the KIV-*VP1* positive cases. This apparent discrepant result might be explained by the nucleotide variability observed in the C-terminal part of *T-Ag* gene of polyomaviruses [Lednicky et al., 1997; White and Khalili, 2004].

Phylogenetic analysis performed on the early region of polyomaviruses included in this study showed the presence of three main clades comprising the BKV, JCV, and KIV strains, respectively. Interestingly, within the KIV clade two sub-clades named A and B were observed. The sub-clade A contained all four Italian isolates, while sub-clade B included all Swedish and Australian strains. Among the Italian isolates, both KIV-RM1 and -RM2 identified in normal tissue (lung tissue surrounding the tumor and paranasal tissue) appeared closely related and clustered together, whereas both the KIV-RM3 and -RM4 strains detected

in lung cancer tissue fell outside this cluster, being more distantly related to KIV-RM1 and -RM2.

Regarding the genetic distance observed between sub-clades A and B, it might be related to the different source of isolation (malignant lung tissue vs. respiratory secretions), and perhaps reflects a different biological behavior of the virus in vivo. To test this hypothesis would be interesting to study the transforming potential of KIV strains in vitro as it is known that the C-terminal part of the T-Ag of polyomaviruses retains binding activity for p53 and Rb proteins. The inactivation of these oncosuppressor proteins by the T-Ag leads to cell cycle deregulation, inhibition of apoptosis, and cell transformation. Therefore, in vitro experiments investigating the transforming potential of KIV isolates are warranted.

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