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Merkel cell polyomavirus DNA in tumor-free tonsillar tissues and upper respiratory tract samples: Implications for respiratory transmission and latency

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Background: Merkel cell polyomavirus (MCPyV) was discovered recently. It is considered a potential causative agent of Merkel cell carcinoma, a life-threatening skin cancer.

Objectives: To study the prevalence of MCPyV in a large number of clinical samples of various types. Most of the samples were examined also for the other newly found polyomaviruses KI (KIPyV) and WU (WUPyV).

Study design: Altogether 1390 samples from immunocompetent or immunocompromised patients, including (i) tonsillar tissues and sera from tonsillectomy patients; (ii) nasopharyngeal aspirates (NPAs) and sera from wheezing children and (iii) nasal swabs, sera and stools from febrile leukemic children were studied for MCPyV. The tonsils, nasal swabs and stools were also studied for KIPyV and WUPyV.

Results: MCPyV DNA was detected in 14 samples altogether; 8 of 229 (3.5%) tonsillar tissues, 3 of 140 (2.1%) NPAs, 2 of 106 (1.9%) nasal swabs and 1 of 840 (0.1%) sera. WUPyV and KIPyV were detected in 5 (2.2%) and 0 tonsils, 1 (0.9%) and 4 (3.8%) nasal swabs and 0 and 2 (2.7%) fecal samples, respectively. The patients carrying in tonsils MCPyV were of significantly higher age (median 42 years) than those carrying WUPyV (4 years, p < 0.001).

Conclusions: MCPyV DNA occurs in tonsils more frequently in adults than in children. By contrast, WUPyV DNA is found preferentially in children. MCPyV occurs also in nasal swabs and NPAs, in a frequency similar to that of KIPyV and WUPyV. The tonsil may be an initial site of WUPyV infection and a site of MCPyV persistence.

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immunocompetent and immunosuppressed individuals. For comparison, we studied the samples also for the two other newly found polyomaviruses KIPyV and WUPyV.

3. Study design

The clinical material comprised nasal swabs, sera and fecal samples from 51 febrile children with acute lymphoblastic leukemia undergoing anticancer treatment (group 1)\(^2\); nasopharyngeal aspirates (NPAs) and paired sera from 140 and 248 wheezing children, respectively (group 2)\(^3-6\); and matched pairs of tonsillar tissue and sera from 229 asymptomatic children or adults (group 3)\(^7\) (Fig. 1). The children of groups 1 and 2 have been studied extensively for 14 and 16 respiratory viruses, respectively, including adenovirus; coronaviruses 229E, OC43; rhinoviruses; enteroviruses; human bocavirus (HBoV); influenza A and B viruses; human metapneumovirus and parainfluenza viruses 1–3. The tonsillar tissues were obtained during tonsillectomy due to chronic tonsillitis or tonsillar hypertrophy. Total nucleic acids were extracted from the sera, tonsillar tissues, nasal swabs and NPAs with the DNA Mini kit (Qiagen, Crawley, UK), and from the fecal samples with QIAamp Qiagen DNA Stool kit (Qiagen) according to the manufacturer’s instructions. The tonsillar tissues and fecal samples were examined individually whereas the sera, NPAs and nasal swabs were initially studied in pools of five, followed by individual examination of positive pools. A negative control of molecular biology grade water was extracted and included in the PCR between every 10 samples.

For MCPyV detection, nested PCR was performed using as outer primers the previously described LT3 primers\(^1\) and as inner primers a pair hybridizing to conserved regions within the LT3 region (LT3iF 5′-TGACCTGGGGAGAGTTTTTG-3′; LT3iR 5′-GAGAAAGAATTAGGTCTTAAAC-3′). The 25-μl amplification reaction consisted of 1× GeneAmp PCR buffer I (Applied Biosystems, Warrington, UK), each dNTP at 0.2 mM, 20 pmol each primer, 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 μl of sample. To increase the robustness of fecal PCRs, BSA (New England Biolabs, Ipswich, USA) included at a final concentration of 0.1 μg/μl. The cycling conditions for the first and the second PCRs were 5 min at 95 °C, followed by 40 cycles of amplification (95 °C for 17.5, 58 °C for 20 s and 72 °C for 20 s). All samples positive for MCPyV were reanalyzed with the LT1/M1 nested PCR primer set as described\(^8\) and sequenced. Samples positive with the LT3 nested primer set but negative with the LT1/M1 primer pair were analyzed with a third (seminested) PCR. Primers LT3sF (5′-CTAAGTGCCCGTTGATAGCTGAAG-3′) and LT3 antisense were used for the first-round PCR whereas the internal primers LT3sF and LT3sR were used for the second-round PCR. The mastermix and cycling conditions were as for the other two PCRs. WUPyV and KIPyV detection was performed by using primer set A as described.\(^9\) All MCPyV and KIPyV/WUPyV PCR products of correct size were sequenced for confirmation of specificity. For use as positive controls and to determine assay sensitivities by limiting dilution analysis, plasmids containing the VP2 gene of WUPyV (EU693907) and KIPyV (EU358767) and the LT3 amplification product of MCPyV (EU375803) were constructed; the amplification products of the VP2 genes were cloned into pCR8/GW/TOPO (Invitrogen; Carlsbad, CA, USA) whereas the MCPyV LT3 region was synthesized and cloned into pcDNA4 by Gene Oracle, Inc. (San Leandro, USA). In the MCPyV and KIPyV/WUPyV assays, plasmid controls with 30 and 5 copies/reaction were reproducibly positive, respectively. Of note, the sensitivities were unaffected by the inclusion of genomic human DNA from cultured 293T cells at 100 ng per reaction (4 ng/μl). In non-nested format with 40 PCR cycles the LT3 primers had a sensitivity 1 log lower than that of the nested assay both in the presence and absence of human genomic DNA.

Three NPAs testing positive for MCPyV DNA were selected for DNase treatment to determine the encapsidation status of the viral DNA. The NPAs were centrifuged at 16,000 × g for 10 min whereafter both the supernatants and the pellets (after resuspension and sonication) were diluted 1:1 with molecular biology grade water and treated with 100 units of DNase I (Roche, Mannheim, Germany) or mock treated with water. After 2 h at 37 °C, the enzyme was inactivated with 8 mM EDTA and heating at 70 °C for 10 min. DNA was isolated with DNA Mini kit (Qiagen).

4. Results

The three novel polyomaviruses, MCPyV, KIPyV and WUPyV, were detected in the nasal swabs of the leukemic children and NPAs of the wheezing children at frequencies of 0.9–3.8% (Fig. 1). Sera from children with MCPyV DNA in NPA were PCR-negative. Two of the 75 fecal samples (2.7%) tested positive for KIPyV DNA whereas all tested negative for MCPyV and WUPyV. Of the 840 sera studied for MCPyV, only one from a leukemic child was PCR-positive. The WUPyV/KIPyV screening of NPAs and sera from the wheezing children had been done in a prior study (manuscript in submission). For MCPyV the highest frequency of viral DNA detection in any sample type was in the tonsillar tissues; in this group of 229 subjects the ages of the patients with MCPyV or WUPyV DNA in tonsils were 20; range 2–72) and 23 years (median 21; range 2–72), respectively, whereas the average ages of the patients PCR-negative for the two viruses were 22 years (median 20; range 2–72) and 23 years (median 21; range 2–72), respectively. The ages of the patients with MCPyV or WUPyV DNA in tonsils was ranked\(^6\) and compared with the unequal variance t-test, yielding a statistically significant difference of \(p < 0.001\) (Fig. 2). Also the differ-

![Fig. 1. Patient and sample groups. Data are numbers (%) of PCR-positive samples. Only samples testing positive for MCPyV with at least two different PCRs and subsequent sequencing are included.](image-url)
sensitivity of the assay. The number of LT1/M1 positive samples was probably due to the lower positive and are shown in Fig. 1. Although not shown, the lower MCPyV positive with two different PCRs were considered MCPyV two remained negative. All PCR products were sequenced for con-
tinuous in the median ages of the patients with and without MCPyV (p = 0.032) or WUPyV (p < 0.001) in tonsil was statistically signifi-
cance. Serum samples from the tonsillectomised patients collected at the time of operation were PCR-negative for the three viruses. Most of the tonsillar MCPyV findings were from patients with chronic tonsillitis or tonsilar hyperplasia, 5/108 (4.9%) and 3/42 (7.1%), respectively. WUPyV occurred in patients with hyperplastic palatine or adenoid tonsillitis (2 of 25, 8%), chronic tonsillitis (2 of 108, 1.9%), and chronic pansinusitis (1 of 2, 50%).

All three wheezing children with MCPyV DNA in NPA (aged 1.7, 1.2 and 4.2 years) showed co-infections with other respiratory viruses (HBoV, rhinovirus and enterovirus along with respiratory syncytial virus (RSV)). Of the two leukemic children with MCPyV DNA in NPA (aged 7 and 4 years), one tested positive for influenza A and RSV antigens whereas the other remained negative in tests for 12 respiratory viruses. In addition to fever, both had cough and rhinitis.

Among the 16 samples that, in our entire study, were positive for MCPyV by using the LT3 nested PCR, 13 tested positive also with the LT1/M1 nested PCR. Of the three LT3 nested PCR-positive samples that were negative with the LT1/M1 nested PCR, one tested positive with the LT3 seminested PCR whereas the other two remained negative. All PCR products were sequenced for con-
firmation of specificity. Throughout our study, only samples testing MCPyV positive with two different PCRs were considered MCPyV positive and are shown in Fig. 1. Although not shown, the lower number of LT1/M1 positive samples was probably due to the lower sensitivity of the assay.2

Three of our MCPyV-positive NPAs were treated (before DNA iso-
lation) with DNase I. Two were reproducibly resistant to the enzyme with no change in the level of viral DNA. One sample was in three repeated assays fully susceptible to DNase I (PCR-negative versus PCR-positive), suggesting that the viral DNA occurred in nonencapsidated form.

5. Discussion

In this study MCPyV DNA was detected in clinical samples of many different types: nasal swabs and nasopharyngeal aspirates, tumor-free tonsillar tissues and sera. Among immunocompetent children, the absence of MCPyV from the 496 serum samples tested is in accordance with our recent study (in submission) in which the two other polyomaviruses KIPyV and WUPyV were absent from all of these sera. Interestingly, our literature survey did not reveal any evidence of occurrence of human polyomavirus DNA in serum or plasma of immunocompetent individuals. On the contrary, blood cells of immunocompetent individuals have been shown to harbor BKV and JCV DNA in several studies. The reported rates of BKV detection in lymphoid cells have been 0%, 10, 26%, 11 53% 12 and 94% 13 and those of JCV 0%, 10 1%, 11 10%,14 and 83%. BKV and JCV DNA have also been reported in the sera or plasma of immunocompromised patients.15,16

Among the three NPAs of wheezing children positive for MCPyV, the invariable occurrence of co-infections (100%) with respiratory viruses may indicate merely an “innocent bystander” role for MCPyV in respiratory disease, as previously suggested for KIPyV and WUPyV.8 Further studies are needed to determine whether MCPyV has any contribution to respiratory tract symptoms.

A recent study reported an apparently higher prevalence of KIPyV (8%) than of WUPyV (1%) among respiratory specimens of 200 patients with respiratory symptoms, 89% of whom were immunocompromised.17 In line with this, we found KIPyV in 3.5% of nasal swabs and 2.7% of fecal samples from the febrile leukemic children whereas WUPyV was found in only 0.9% and 0% of the samples, respectively. Of note, WUPyV has been reported in 2 of 377 (0.5%) fecal samples from immunocompetent children with acute gastroenteritis.18

Two very recent reports have disclosed the presence of MCPyV in NPA samples of predominantly immunocompetent children (<15 years) with an average genoprevalence of 0.9%.19,20 Our study, with even slightly higher MCPyV prevalences of 2% and 3% among immunocompetent and immunocompromised children, respectively, is in line with the other two. Furthermore, we showed widespread occurrence of MCPyV DNA in tonsillar tissue. This may be a general feature of human polyomaviruses, as tonsillar tissues can harbor both JCV17 and BKV22 DNA and tonsillar stromal cells have been shown to be susceptible to JCV infection.23 Blood cell-
derived MCPyV positivity of the tonsils could not be completely ruled out as only serum samples (all of which tested negative for MCPyV DNA) obtained at the time of operation were available from our tonsillectomy patients. The potential presence of MCPyV in blood cells of immunocompetent individuals as opposed to serum is an interesting topic for further research.

The age distribution of our MCPyV-positive biopsy donors is in strong agreement with the study of Goh et al. showing in NPAs Merkel cell virus DNA more frequently in adults (8.5%) than in young children (0.6%). This is very interesting, as in the NPAs of immunocompetent individuals KIPyV and WUPyV occur more frequently in children than adults.24 In light of the equivalent NPA prevalences of WUPyV and MCPyV among immunocompetent children, the notable age difference in tonsillar occurrence of MCPyV versus WUPyV possibly reflects a major dissimilarity in the life cycles of these two viruses, related with persistence versus primary infection. Indeed, our small-scale DNase data suggest that even among children MCPyV DNA in the upper airways is not necessarily encapsidated among all individuals. On the basis of our results and those of Goh et al., showing MCPyV DNA mainly in adults, it is tempting to speculate that the occurrence of MCPyV in the respiratory tract may involve active shedding coupled with respiratory transmission among children, leading to DNA persistence towards adulthood.

Conflict of interest

No conflicts.
Acknowledgments

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