Urinary polyomavirus infections in neurodevelopmental disorders

Ivan Gentile1,2, Laura Altieri3,4, Carla Lintas3,4, Roberto Sacco3,4, Paolo Curatolo5, Arianna Benvenuto5, Filippo Muratori6, Elisa Santocchi6, Carmela Bravaccio7, Carlo Lenti8, Raffaella Faggioli9, Roberto Rigardetto10, Marina Gandione10, Giuseppe Portella7, Emanuela Zappulo1, Guglielmo Borgia1,2, Antonio M. Persico3,4,11

1Department of Clinical Medicine and Surgery, University of Naples “Federico II”, Naples, Italy
2Interdepartmental Center of Research in Basic and Clinical Immunological Science (CISI), University of Naples “Federico II”, Naples, Italy
3Unit of Child and Adolescent Neuropsychiatry, Laboratory of Molecular Psychiatry and Neurogenetics, University “Campus Bio-Medico”, Rome, Italy
4Department of Experimental Neurosciences, I.R.C.C.S. “Fondazione Santa Lucia”, Rome, Italy
5Department of Child Neuropsychiatry, University “Tor Vergata”, Rome, Italy
6Department of Child Neurology and Psychiatry, I.R.C.C.S. “Stella Maris”, University of Pisa, Pisa, Italy
7Department of Translational Medical Science, University “Federico II”, Naples, Italy
8Department of Child Neuropsychiatry, University of Milan, Italy
9Fondazione Teda per l’Autismo ONLUS, Turin, Italy
10Department of Child Neuropsychiatry, University of Turin, Turin, Italy
11Mafalda Luce Center for Pervasive Developmental Disorders, Milan, Italy

Email: carmela.bravaccio@unina.it

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ABSTRACT

We have recently reported enhanced frequencies of polyomavirus infection in post-mortem brain tissue of autistic patients compared to controls. To further explore potential contributions to neurodevelopmental disorders by polyomaviruses, we have employed species-specific TaqMan assays to assess the prevalence and titres of BKV, JCV and SV40 in the urines of 87 patients with autism spectrum disorder, 84 controls matched by sex and age with the autistic sample, 15 subjects with Down syndrome and 13 fragile X individuals. Prevalence rates of urinary BKV infection were significantly greater in Down syndrome and fragile X patients compared to autistic and control individuals (P < 0.01). In a large majority of patients who showed the presence of urinary genomes, viral titres resulted significantly higher among Down syndrome patients (P < 0.01) compared to controls, autism spectrum disorder and fragile X individuals, who did not significantly differ from each other. Our results are consistent with previous evidence supporting hampered immunological surveillance and/or immune deficits in fragile X and especially in Down syndrome patients.

Keywords: Autism; BKV; Down Syndrome; Fragile X Syndrome; JCV; SV40

1. INTRODUCTION

Down syndrome (DS), fragile X syndrome (FXS) and autism spectrum disorder (ASD) represent three common developmental disorders encompassing abnormalities primarily affecting the central nervous system (CNS). DS is caused by a complete or partial trisomy of chromosome 21 [1]. The incidence of DS is directly proportional to maternal age and on average affects 1:750 live births [2]. The disease is associated with mental retardation, congenital cardiac defects, autoimmune disorders (celiac disease, hypothyroidism, type I diabetes mellitus), immunodeficiency with increased incidence of bacterial and viral infections, and early onset Alzheimer’s disease [3-6]. Interestingly, both genome-wide and chromosome 21-targetted expression studies have unveiled the consistent over-expression of only approximately 25% - 30% of trisomic genes, in addition to 3% - 6% of loci distributed on each of the remaining chromosomes [7,8]. DS patients display prominent interindividual variability in clinical phenotype and disease severity, with approximately 8% showing severe autistic behaviours [9].
Fragile X syndrome (FXS) is caused by the expansion of a triplet repeat located in the 5’ untranslated region (UTR) of the FMR1 gene (Xq27.3) [10]. When the normal 30-repeat allele becomes unstable and expands beyond 230 repeats, the FMR1 locus shows hypermethylated and transcription is silenced. Lack of the fragile X mental retardation protein (FMRP), an RNA-binding protein encoded by the FMR1 gene, is responsible for the disease phenotype [11]. The cardinal symptom of FXS is mental retardation and the phenotype is obviously more severe in males, who carry a single FMR1 allele [12]. Also in FXS a single and well-defined genetic abnormality translates into cognitive, behavioural and morphological signs and symptoms displaying significant interindividual variability [12]. In particular, approximately 40% of FXS patients are compatible with a diagnosis of autism, possibly reflecting specific neuroanatomical underpinnings [13].

ASD is characterized by deficits in social interaction and communication, as well as by stereotyped behaviours, with onset prior to 3 years of age [14]. Current psychiatric nosography distinguishes severe forms of the spectrum (“Autistic Disorder”), from conditions where speech is preserved (“Asperger Disorder”) and from milder forms displaying some, but not all of the core symptoms (“Pervasive Developmental Disorder Not Otherwise Specified” or PDD-NOS) [14]. The incidence of ASD has dramatically risen during the last two decades from 2 - 5/10,000 to approximately 2 - 6/1000 children [15,16]. Approximately 10% of ASD patients suffer from “syndromic” autism, i.e., secondary to a known genetic disorder, such as tuberous sclerosis, neurofibromatosis, FXS, and large chromosomal rearrangements, or to identified prenatal teratological agents, such as rubella and cytomegalovirus (CMV) infection, or exposure to drugs including thalidomide, misoprostol, and valproic acid [17-21]. The remaining 90% of patients are affected by non-syndromic or “idiopathic” autism. Altered neurodevelopment occurring during the first and second trimester of prenatal life is recognized as the underlying neuropathological cause of ASD in most patients [22]. Heritability estimates for autism are the highest among all neuropsychiatric disorders (see Discussion), but the genetic underpinnings of autism remain complex and largely elusive. Furthermore, several human studies have pointed toward possible viral contributions to the pathogenesis of ASD, compatible with high heritability and derangement of early prenatal neurodevelopment [23]. In particular, rubella and CMV represent the two infectious agents best-known to enhance autism risk following a congenital infection [23,24]. The largest longitudinal study involving several hundred children prenatally exposed to rubella virus estimates at 7.4% the rate of autism in this group (vs 2 - 6/1000 autism prevalence in the general population); risk appears especially high if the infection occurs during the first 8 weeks post-conception [17]. Evidence linking prenatal CMV infection to autism is more circumstantial: several case reports have been published, but risk estimates are essentially based on a small cohort of 7 prenatally CMV-infected children, who displayed autistic features in 2 cases (2/7 = 28.6%) [21]. It remains to be determined to what extent autism ensues from direct viral damage, from the strong immune response driven by herpes viruses, such as CMV, or from the nature and location of cerebral malformations which are particularly frequent in congenital CMV infection. Autistic children prenatally infected with rubella or CMV typically display “low-functioning” autism, accompanied by severe mental retardation, seizures, and physical anomalies, such as ophthalmologic malformations, deafness, cardiac malformations. Brain imaging findings are highly variable, ranging from normal to cortical malformations (polymicrogyria, pachygyria, heterotopias) indicative of migration defects, to abnormal intensity of the periventricular white matter suggestive of abnormal myelination in the absence of any cortical malformation. These clinical and brain imaging features are not typical of most “idiopathic” autistic children, as prenatal rubella and CMV do not represent frequent causes of autism. Nonetheless this pathogenetic paradigm demonstrates that prenatal viral infections can indeed cause autism and that other viral species could be involved, provided their mechanism is reconciled with high heritability and with an early prenatal onset for deranged neurodevelopment later leading to abnormal behaviour.

We have recently reported enhanced frequencies of polyomaviruses (BKV, JCV and SV40) infection in postmortem neocortical tissue of ASD patients compared to controls [25]. Multiple lines of evidence support vertical transmission of polyomaviruses as a potential cause of autism in a sizable subgroup of patients (see Discussion). In the present study, we assess the prevalence and titres of BKV, JCV and SV40 in the urines of 64 ASD, 15 DS, and 13 FXS patients, as well as 64 controls tightly matched by sex and age with the ASD sample.

2. MATERIALS AND METHODS
2.1. Urine Samples
Urines from 87 ASD children, 15 individuals with DS, and 13 FXS patients were collected. Tight sex- and age-matching (±2 yr) to the ASD sample was applied to recruit 84 typically developing controls among the offspring of staff members devoid at the time of any overt sign or symptom of illness. The demographic characteristics of the four experimental groups are summarized in
Urine samples were stored at −80°C until analysis. Viral DNA was extracted from 140 µl of urine, using a commercially available kit (QIAamp Viral RNA Minikit, Qiagen, Inc.), according to the manufacturer’s instructions.

2.2. Real Time PCR

Urinary titres of viral DNA were quantified by real time PCR using appropriate TaqMan assays (Applied Biosystems, Foster City, CA). Briefly, a standard curve was prepared for each assay using a serial 10-fold dilution of plasmids containing the appropriate viral genome (range 0 - 10^8 copies). Each sample was assayed in triplicate. BKV, JCV or SV40 viral loads are expressed as copies/µl of urine. A BKV or JCV gene sequence from the VP1 region and an SV40 gene sequence from the early coding region were amplified using the primers and probes listed in Table 2. TaqMan probes were labelled with FAM and TAMRA at 5' and 3', respectively. PCR cycling conditions for all viral species were 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. PCR was performed in a total volume of 25 µl, including MasterMix Genotyping 2x (Applied Biosystems, Foster City, CA), 400 nM of each primer (Invitrogen, Carlsbad, CA) and 200 nM probe (Applied Biosystems, Foster City, CA). The sensitivity of TaqMan assays for BKV and JCV was at 100 copies/µl, whereas SV40 was at 10 - 100 copies/µl; all assays were completely specie-specific, except for the BKV assay which amplifies JCV at high titres (10^5 - 10^8 copies/µl) not recorded in any individual assessed here.

2.3. Statistical Analysis

Prevalence rates of polyomavirus infection in urines were compared using the X^2 test and Fisher’s exact test, where appropriate. Kolmogorov-Smirnov test was applied to check for Gaussian distribution of quantitative variables. In case of Gaussian distribution mean and SD were used to present data and ANOVA test for comparing variables. In case of non-Gaussian distribution, median and interquartile range (IQR) were used to present data and Kruskal-Wallis test was employed to compare them. Two-tail P values are reported throughout the manuscript.

3. RESULTS

The prevalence of urinary Polyomavirus infection in ASD, DS, FXS patients and controls is shown in Table 3. DS and FXS patients display greater-than-double prevalence rates of urinary BKV infection compared to ASD and controls, as BKV is present in 8/15 (53.5%) DS and 8/13 (61.5%) FXS individuals vs 21/84 (25%) controls and 16/87 (18.4%) ASD patients (X^2 = 16.718, 3 df, P = 0.001).

Urinary BKV titres, measured by specie-specific TaqMan assay in 15 ASD, 8 FXS, 8 DS, and 17 controls positive for this virus are significantly different among the four diagnostic groups (K-W X^2 = 11.60, 3 df, P = 0.009). DS individuals show 2 - 3 times higher urinary BKV titres compared to the other three diagnostic groups, which do not differ from each other (DS vs controls, U = 14, P = 0.002; DS vs ASD: U = 18, P = 0.007; DS vs FXS, U = 13, P = 0.046).

Urinary titres of BKV did not differ significantly between ASD and control group. Prevalence rates of urinary JCV and SV40 infection did not differ among ASD, DS, FXS patients and controls (Table 3). These two species were found in 14 out of a total of 199 individuals recruited for this study: the small number of positive samples hindered meaningful quantitative analyses of urinary viral titres for JCV and SV40.

4. DISCUSSION

The present study reports enhanced rates of urinary BKV infection in DS and FXS patients compared to controls and autistic individuals, as well as significantly elevated urinary BKV titres in DS patients. Our experimental approach appears both reliable and valid. The TaqMan assays used to quantify urinary BKV, JCV, and SV40 titres were previously described [26-28], and have yielded in our hands sensitivities and specificities superimposable to those reported in the original publications. Each urine sample was analysed in triplicate and the four diagnostic subgroups were always assayed in parallel. The higher prevalence of urinary infection by BKV compared to JCV was initially unexpected, as the vast majority of studies involving immunocompetent individuals indeed show the opposite trend [29]. However, these studies typically
Table 2. Primers and probes used in TaqMan assays to quantify viral titres.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer/Probe Name</th>
<th>Primer/Probe Sequence (5’ - 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1 Din For</td>
<td>TGCTGATATTTGTGGGCTGT TACTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BKV</td>
<td>VP1 Din Rev</td>
<td>CTCAGCGGATCTTTAAAAATATCTTG</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>VP1 Din Probe</td>
<td>6-FAM-AGCTCTGGAACACAAACAGTGAGAAGGAGC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>JC</td>
<td>JC VP1 F bis</td>
<td>CTCAATGGATGTGCTTTTACTTT</td>
<td></td>
</tr>
<tr>
<td>JCV</td>
<td>JC VP1 R bis</td>
<td>CGGGTGTCCCTTCTTCTCC</td>
<td>mod. from [27]</td>
</tr>
<tr>
<td></td>
<td>JC VP1 Probe</td>
<td>6-FAM-AGGTGTACGGACTGTAACCTGCTC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>SE2</td>
<td>SE2 early For</td>
<td>GTGGAATGCGCTTTAAATGAGGAA</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>SE2 early Rev</td>
<td>TTGAGATGCAGTACGCTCTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SE2 early Probe</td>
<td>6-FAM-CCTGTTTTTGCTCAGAAGAATGCCCATCTAGTG-TAMRA</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Prevalence of urinary BKV, JCV and SV40 infection.

<table>
<thead>
<tr>
<th></th>
<th>Controls (N = 84)</th>
<th>ASD (N = 87)</th>
<th>Down (N = 15)</th>
<th>FXS (N = 13)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKV+</td>
<td>21 (25.0%)</td>
<td>16 (18.4%)</td>
<td>8 (53.3%)</td>
<td>8 (61.5%)</td>
<td>(X^2 = 16.718, 3 \text{ df}, P = 0.001)</td>
</tr>
<tr>
<td>BKV−</td>
<td>63 (75.0%)</td>
<td>71 (81.6%)</td>
<td>7 (46.7%)</td>
<td>5 (38.5%)</td>
<td></td>
</tr>
<tr>
<td>JCV+</td>
<td>3 (3.6%)</td>
<td>2 (2.3%)</td>
<td>0 (0%)</td>
<td>2 (15.4%)</td>
<td></td>
</tr>
<tr>
<td>JCV−</td>
<td>81 (96.4%)</td>
<td>85 (97.7%)</td>
<td>15 (100%)</td>
<td>11 (84.6%)</td>
<td></td>
</tr>
<tr>
<td>SV40+</td>
<td>’3 (4.7%)</td>
<td>’1 (1.6%)</td>
<td>2 (13.3%)</td>
<td>1 (7.7%)</td>
<td>(X^2 = 4.33, 3 \text{ df}, P = 0.23, \text{n.s.})</td>
</tr>
<tr>
<td>SV40−</td>
<td>”61 (95.3%)</td>
<td>”63 (98.4%)</td>
<td>13 (86.7%)</td>
<td>12 (92.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Note: + = present, − = absent, N = 64.
cytokine and chemokine levels [43], but much fewer data are available in the literature for FXS compared to DS in this regard.

ASD has been identified by family and twin studies as the most “heritable” neuropsychiatric disorder, with concordance rates of 82% - 92% in monozygotic (MZ) twins vs 1% - 10% in dizygotic (DZ) twins; sibling recurrence risk is 3% - 6% for strict autism and approximately 15% for broad ASD, much higher than prevalence estimates in the general population [20,44-46]. Yet, two decades of genetic investigation have unveiled relatively few cases which can be solely explained on the basis of de novo mutations or cytogenetic abnormalities [47]. We have thus hypothesized that vertical viral transmission from parents to offspring mediated by parental gametes may play a role in autism [25,48]. In our recent post-mortem study, the presence of polyomavirus genomes in neocortical tissue was significantly associated with an ASD diagnosis [25]. Converging lines of evidence, obtained using these same brain tissues either directly or indirectly, support an inappropriate and persistent activation of the innate immune system in ASD, compatible with an early unresolved viral infection in the CNS of many autistic individuals [49-51]. Furthermore, several ASD children display immunological abnormalities, which could point toward abnormal handling of viral agents by the immune system [52,53]. Strikingly, as many as 48% of ASD children display MRI abnormalities strongly resembling CNS viral infections, especially around the temporal lobes [54]. Also the HGF/MET pathway has been demonstrated to undergo strong activation during SV40 replication and the gene encoding MET is significantly associated with autism [55,56].

The presence of genome in urine of immunocompetent subjects is only an indicator of current infection or viral reactivation, not a reliable marker of life-time exposure. In this regard, the higher rate of BKV genome detection in patients with DS, which is associated with marked immune deficits, is consistent with a reactivation from such viruses. The potential contribution of infection/reactivation by polyomavirus in shaping the clinical phenotype of these neurogenetic syndromes remains unknown.

On the other hand, ASD patients do not differ here from controls in prevalence rate of urinary polyomavirus infection and in viral titres. This negative finding is not necessarily incompatible with prenatal or early postnatal roles of polyomaviruses in ASD, since congenital infections by viral agents, such as CMV and HSV1, does not largely result in persistent or recurrent shedding later in life, and the duration of viral excretion is not correlated with long term outcome [57,58]. We are thus testing our hypothesis by directly assessing the prevalence of genomic infection in parents of ASD children. Also serological assays able to detect virus-specific serum antibodies should provide an estimate of lifetime exposure rates in ASD and control subjects. These two experimental approaches should jointly provide a conclusive test of the possible role of polyomavirus infections which, occurring in a very early embryological period, could be involved in the pathogenesis of these disorders.

5. ACKNOWLEDGEMENTS

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