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Polyomavirus JC reactivation and noncoding control region sequence analysis in pediatric Crohn's disease patients treated with infliximab

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Abstract The recent introduction of monoclonal antibodies in Crohn's disease (CD) management has been associated with the development of serious complications, such as the progressive multifocal leukoencephalopathy (PML), caused by JC polyomavirus (JCV) reactivation. Therefore, the aims of our study have been the investigation of the possible JCV reactivation in pediatric CD patients treated or not with infliximab, performing quantitative PCR in urine, plasma, and intestinal biopsies at the time of recruitment (t0) and every 4 months in 1 year of follow-up (t1, t2, and t3), and the analysis of the JCV noncoding control region (NCCR) to detect cellular transcription factors binding site mutations. Results obtained showed that, in urine and ileal specimens, JCV load significantly increased in infliximab-treated patients after 1 year of treatment (t3), while viremia was significantly higher at t1. JCV NCCR sequence analysis showed a structure similar to CY archetype in 65/80 analyzed sequences, but the remaining 15/80, obtained exclusively from plasma and biopsies, evidenced a CY NCCR organization with two recurrent nucleotide changes, the 37-T to G transversion in box A Spi-B binding site and the 217-G to A transition in box F, and a box D deletion. These rearrangements were always found at t3 within seven infliximab-treated CD patients, who presented a very severe disease at t0. We can conclude that our rearranged NCCR sequences could be considered a marker of JCV virulence during mAb treatment, although none of our examined patients developed PML, and further studies on a larger cohort of patients should be performed.

Keywords Crohn's disease · Infliximab · Human polyomavirus JC · Noncoding control region · Spi-B · PML

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Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD), with increasing prevalence that reflects the interaction of at least three components: a genetic predisposition, an environmental trigger, and an unregulated or deregulated immune response (Bellizzi et al. 2010; Comerford and Bickston 2004; Mayer 2010). Various medications, including 5-aminosalicylates, antibiotics, corticosteroids, and immune-modulators, such as methotrexate, have traditionally been used to control inflammation. Recent introduction of the monoclonal antibodies (mAbs) in the management of CD [such as the tumor necrosis factor-α (TNF-α) inhibitors infliximab (Remicade, Centocor Ortho Biotech) and adalimumab (Humira, Abbott)] has been immediately associated with the development of serious

life-threatening infections. Infliximab is a specific chimerical anti-TNF-α monoclonal antibody (mAb) constituted by the constant region of the human immunoglobulin G1k (IgG1k) linked to the variable antigen-binding regions of a murine anti-human TNF-α antibody (Knight et al. 1993; Sandborn 2001). The TNF-α blocking results in decreased expression of interleukin-1 and interleukin-6, interferon-γ, and other pro-inflammatory cytokines (Hansel et al. 2010; Sandborn and Targan 2002). Although infliximab reduced clinical symptoms, it also could unbalance the local immune-surveillance and enhance the latent virus reactivation (Bellizzi et al. 2010). Among the main latent viral infections, the human polyomavirus JC (JCV) reactivation in CD after mAb therapy and its association with progressive multifocal leukoencephalopathy (PML) have been reported after using natalizumab (Tysabri, Biogen-Idec, Elan Pharmaceuticals) (Major 2010; Marshall and Major 2010). In November 2004, natalizumab was approved by the US Food and Drug Administration for the treatment of relapsing-remitting multiple sclerosis (MS), but it was suspended in February 2005 on the discovery of three cases of PML (Kleinschmidt-DeMasters and Tyler 2005; Langer-Gould et al. 2005; van Assche et al. 2005). Natalizumab was reintroduced in July 2006 as second-line monotherapy for MS with specific warnings and precautions, including the TOUCH Prescribing Program (TOUCH® 2010) to minimize the risk of PML. By mid-2006 until November 2009, there were a total of 28 cases of confirmed PML in patients with MS treated with natalizumab (Clifford et al. 2010). Although anti-α4 integrin therapy with natalizumab is efficacious in refractory CD and in MS, screening for the risk of PML has not been developed (Hansel et al. 2010; Verbeeck et al. 2008). Recently, it has been reported the first case of PML in a man with erosive rheumatoid arthritis after 3 years of treatment with infliximab (Kumar et al. 2010). PML is a fatal neurodegenerative demyelinating disease that is generally due to reactivation of latent infection of JCV in the central nervous system (CNS). PML is characterized by multiple regions of demyelination, which are developed upon lytic infection of oligodendrocytes, the myelin-producing cell of the CNS, by JCV (Berger 2007; Khalili et al. 2008). Although JCV is ubiquitous among population with greater than 80% of adults exhibiting JCV-specific antibodies, only individuals with severely impaired immunity, mainly AIDS patients, can develop PML (Major 2010). Despite its lytic capability, JCV can persist in a variety of cell types including CD34+ hematopoietic precursors and B cells present in bone marrow, brain, tonsil, and in circulation (Tan et al. 2009). Trafficking of JCV-infected B cells, or hematopoietic precursors, between the bone marrow and brain is a possible method of viral dissemination (Marshall et al. 2010).

The JCV genome is divided into an early and a late region that are physically separated by the noncoding viral control region (NCCR) (Frisque et al. 1984; Mischitelli et al. 2010). NCCR is the most highly variable sequence among JCV isolates but always contains the origin of replication, one or more TATA boxes and a variety of enhancer elements (Ault and Stoner 1993; Frisque et al. 1984; Marshall et al. 2010). The NCCR of a naturally occurring variant of JCV shed in urine of healthy patients, referred to as nonpathogenic archetype (CY), is divided into six regions named box A (36 bp), B (23 bp), C (55 bp), D (66 bp), E (18 bp), and F (69 bp) (Frisque et al. 1984; Mischitelli et al. 2005). Each region contains binding sites for transcriptional cell factors involved in viral early and late transcription. These binding sites undergo deletion and enhancement process that could generate variants that could up-modulate viral expression in a specific anatomical site. In fact, the NCCR isolated from the brain of a patient with PML (strain Mad-1) contains a promoter/enhancer that exists as 98-bp tandem repeats of the boxes A (36 bp), C (55 bp), and E (18 bp), including two TATA boxes and multiple cellular transcription factor-binding sites, and the deletion of the boxes B and D (Frisque 1983; Marshall et al. 2010). Recently, some authors have focused their attention on the binding site for the cellular transcription factor Spi-B, localized within the box A of both CY and Mad-1 NCCR sequences (Lindberg et al. 2008; Marshall et al. 2010). Since Spi-B is an activator of JCV gene expression, it could be a contributing factor in the development of PML in patients undergoing these immunomodulatory therapies (Marshall et al. 2010). In fact, Spi-B is upregulated in peripheral blood mononuclear cells in response to treatment with natalizumab (Major 2010).

Since PML could be a class effect of the new mAbs, the aims of our study have been (1) the JCV detection by real-time PCR (Q-PCR) in urine and plasma specimens and intestinal biopsies obtained from a pediatric cohort of standard therapy responders CD patients and from a pediatric cohort of standard therapy nonresponders CD patients and then treated with infliximab at the time of recruitment and every 4 months from treatment starting. In the intestinal biopsies, the JCV load was monitored only 1 year from treatment starting; and (2) the study of the possible rearrangements of JCV NCCR in order to detect cellular transcription factors binding site mutations.

Materials and methods

Enrolled patients characteristics and study schedule

In this perspective observational study, two cohorts of pediatric patients, referred to the Pediatric Gastroenterology

and Liver Unit of *Sapienza* University of Rome, from January 2010 to January 2011, were enrolled (Table 1):

1. Cohort of 18 patients (mean age 15 years old) with refractory active CD for whom an induction regimen of infliximab was administered, considering a luminal or fistulizing disease refractory to standard treatment, according to approved indications (*cohort 1*) (Table 1). Inclusion criteria were CD diagnosis confirmed with usual clinical, endoscopic, radiologic, and/or histologic criteria for at least 6 months; and active luminal or fistulizing disease despite adequate standard treatments. Exclusion criteria were contraindications to infliximab treatment, such as a history of tuberculosis or positive skin test/chest radiogram in the absence of adequate antibiotic prophylaxis, congestive heart failure, demyelinating syndrome, sepsis or abscesses, symptomatic bowel stenoses, history of previous or present cancer, or failure to give informed consent.
2. Cohort of 25 CD patients (mean age 14 years old), who underwent to standard therapy (*cohort 2*) (Table 1). Inclusion criteria were CD diagnosis confirmed with usual clinical, endoscopic, radiologic, and/or histologic criteria for at least 6 months, and absence of active luminal or fistulizing disease after the use of an adequate standard treatments.

After enrollment, clinical characteristics of CD disease were recorded (including concomitant treatments), clinical activity was measured with the Pediatric CD Activity Index (PCDAI), collected retrospectively during the visit immediately before infusions according to the Frenz et al. protocol (Frenz et al. 2005), and fistula were assessed according to the Present et al. (Present et al. 1999) assessment system. The therapeutic program of the patients

Table 1 Features of the enrolled patients

Enrolled patient cohorts			
	cohort 1[b]	cohort 2[c]	control cohort[d]
No. of patients	18	25	10
Males	10	14	4
Females	8	11	6
Mean age	15	14	12
PCDAI[a]	>30	10–30	–

[a] At the moment of the enrolment, all patients were assessed through the Pediatric Crohn's Disease Activity Index (PCDAI) (score ≤10: inactive disease; 10–30: mild disease; >30: moderate to severe disease)

[b] Cohort of standard therapy nonresponders CD patients and then treated with infliximab

[c] Cohort of standard therapy responders CD patients

[d] Cohort of control patients affected by not IBD intestinal inflammatory

of the *cohort 1* consisted of three consecutive infusions of infliximab (5 mg/kg) at 0, 2, and 6 weeks for the induction phase. The first infusion was given within 7 days of enrollment into the study. Infliximab was administered intravenously during a period of 2 h, and patients were monitored for heart frequency and for signs of adverse reactions. From each patient, blood samples were taken before infusion, after 8 weeks the beginning of therapy and every 8 weeks thereafter, so as to determine the activity and nutritional indexes as well as serum variables of renal and hepatic function. All patients were offered a maintenance therapeutic program consisting of repeated infusions of infliximab every 8 weeks for 1 year. The first re-treatment infusion was administrated 8 weeks after the last baseline infusion.

Clinical effectiveness was evaluated at week 6: remission was defined by a PCDAI score of less than 10 (for luminal disease) or if all fistulae openings present at baseline were closed (for fistulizing disease). Improvements were determined based on the physician's global evaluation of amelioration compared with baseline (Lavagna et al. 2007).

Finally, as control group, we enrolled 10 patients (mean age 12 years old) affected by non-IBD intestinal inflammation (*control cohort*) (Table 1).

Sample collection

We collected 172 plasma (on a routine 4-mL EDTA tube collecting) and 172 urine samples, 72 from *cohort 1* and 100 from *cohort 2*, at the following times: at the time of recruitment (t0) and at 4, 8, and 12 months from t0 (t1, t2, and t3 respectively). Moreover, 86 ileal and 86 colon–rectal biopsies were recruited only at t0 and at t3 within both cohorts (36 for *cohort 1* and 50 for *cohort 2*). For the control group, 10 plasma, 10 urine samples, 10 ileal, and 10 colon–rectal biopsies were obtained from patients affected by not IBD intestinal inflammatory (*control cohort*), only at the moment of diagnosis (t0).

Plasma, urine, and biopsy specimens (15 mg of each) were utilized for DNA extraction procedure by DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

JCV T-Ag real-time PCR (Q-PCR)

Extracted DNA of each samples was analyzed using Q-PCR for the detection and quantification of the JCV genome using a 7300 Real-Time PCR System (Applied Biosystems, USA), following a published protocol (Delbue et al. 2008). Of DNA, 500 ng was used as a template in each reaction, and a 54-bp amplicon in the JCV T antigen region was detected. Each sample was analyzed in

duplicate, and the viral load results were given as the mean of the two positive reactions. Each run contained a negative control composed of the reaction mixture without DNA template. A positive control consisted of serial dilutions (range, 10^5 copies/ml–10^2 copies/ml) of a plasmid containing the entire JCV genome, on the results of which a standard curve was performed. For urine and plasma specimens, the JCV DNA load was expressed as genome equivalents (Geq)/ml of sample and as genome equivalents (Geq)/10^5 cells of sample for the biopsies. To correct for the variable amount of DNA in different tissue samples, each sample was subjected to simultaneous TaqMan PCR for the housekeeping gene Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, Accession no. J04038), targeting the region between exons 6 and 8. Results were considered acceptable only in the presence of GAPDH positivity (Costa et al. 2009).

JCV NCCR PCR

DNA yield was determined by measuring its concentration in the eluate by absorbance at 260 nm, and then, 0.1 to 1 μg of total DNA was directly used in PCR amplification. β-Globin PCR was performed on extracted DNA to assess the efficacy of nucleid acid extraction (Saiki et al. 1985). General precautions, conditions for PCR analysis, and nested-PCR procedures were performed as published (Pietropaolo et al. 2003). β-Globin-positive samples were amplified in a GeneAmp® PCR System 9700 (Perkin-Elmer Cetus, Emeryville, CA), and all assays included positive (purified recombinant plasmid DNA) and negative (all the PCR components except the template) controls to exclude false-positive and false-negative results (Kwok and Higuchi 1989). Nested-PCR employed two pairs of primers that anneal to the invariant regions flanking the NCCR of JCV (Pietropaolo et al. 2003). Primers BKTT1 (5′-AAG GTC CAT GAG CTC CAT GGA TTC TTC C-3′) and BKTT2 (5′-CTA GGT CCC CCA AAA GTG CTA GAGCAG C-3′) amplified a 724-bp DNA fragment in JCV (Mad-1) (Flaegstad et al. 1991). The second pair, JC1 (5′-CCT CCA CGC CCT TAC TAC TTC TGA G-3′) and JC2 (5′-AGC CTG GTG ACA AGC CAA AAC AGC TCT-3′), amplified a portion of the first round PCR product, generating a fragment of 308 bp (Markowitz et al. 1993). Two nanograms of recombinant pGem-1 plasmid DNA containing the complete JCV genome, cloned as EcoR1 fragments, were used as positive controls. The PCR products were analyzed on 2% agarose gels by ethidium bromide staining.

JCV VP1 PCR

For JCV genotyping a 215 bp fragment was amplified from the VP1 major capsid protein gene using primers JLP-15 (5′-ACA GTG TGG CCA GAA TTC ACT ACC-3′) and JLP-16 (5′-TAA AGC CTC CCC CCC AAC AGA AA-3′) (Agostini et al. 2001). After initial denaturation at 95°C for 9 min, followed by 40 cycles at 95°C for 40 s, annealing at 63°C for 40 s and extension at 72°C for 40 s; the amplification protocol was concluded with a final extension at 72°C for 7 min. PCR products were analyzed using 2% agarose gel electrophoresis and visualized using ethidium bromide staining (Mischitelli et al. 2010).

Sequencing of JCV NCCR and VP1 regions

The PCR product corresponding to JCV NCCR and VP1 regions were purified prior to sequencing to remove the excess of primers with QIAquick PCR purification kit, according to QIAGEN protocol (Pietropaolo et al. 2003). DNA sequencing was performed by automatic DNA sequencer (Applied Biosystem, model 370 A), according to the manufacturer′s specifications (Amplicycle Kit, Applied Biosystem). Sequences were organized and analyzed using the Genetic Computer Group Sequence Analysis software package.

Data analysis

Data were summarized as medians and ranges or as mean, as appropriate. If Z test indicated a nonnormal distribution, we used nonparametric test such as Mann–Whitney U tests and Kruskal–Wallis tests. Categorical data were analyzed by using χ^2 test and Student's t test. P values <0.05 were considered statistically significant.

Results

The Q-PCR analysis on our collected samples highlighted the presence of JCV in 80/216 samples within *cohort 1* and in 51/300 samples within *cohort 2*, with a significant association between the two cohorts ($p<0.05$). Table 2 shows in detail the JCV positivity distributed among the various types of samples analyzed in our study. In particular, the total number of positive samples was significantly higher in *cohort 1* than in *cohort 2* in urine ($p=0.03$), plasma ($p<0.05$), and colon–rectal biopsies ($p=0.02$) (Table 2). All examined control group samples resulted negative to the presence of JCV (data not shown).

Regarding the follow-up of the JCV detection in urine, the viral DNA was found in 6/18 urine of *cohort 1* and in 7/25 urine of *cohort 2* at t0. Four months later (t1), the number of patients with viruria increased up to 10 in both cohorts (10/18 and 10/25, respectively), whereas at t2, a reduction of JCV viruria was observed (6/18 in *cohort 1* and 4/25 in *cohort 2*). Finally, at t3, we found JCV in 6

Table 2 Total number of positive and negative samples for JCV DNA

Cohorts	Type of samples														
	Urine			Plasma			Ileum			Colon–rectum			Total		
	Pos	Neg	Tot	Pos	Neg	Tot	Pos	Neg	Tot	Pos	Neg	Tot	Pos	Neg	Tot
cohort 1	28	44	72	23	49	72	17	19	36	12	24	36	80	136	216
cohort 2	24	76	100	7	93	100	14	36	50	6	44	50	51	249	300
p^a	0.03			<0.05			0.07			0.02			<0.05		

[a] By χ^2 test

urine out of 18 CD patients of *cohort 1* and in 3 urine out of 25 CD patients of *cohort 2* (Table 3). No significant association between the number of patients with JCV viruria was found in the two cohorts during the follow-up ($p>0.05$) (Table 3). Comparing the urinary viral loads of the two examined cohorts, we observed that viruria significantly increased in the patients treated with infliximab respect to the patients receiving a standard therapy only at t3 ($p=0.039$), as shown in Table 3. In fact in *cohort 2* the median viral load at t3 was 5.36 log GEq/mL, whereas in *cohort 1*, it reached the value of 7.47 log GEq/mL (Table 3). However, when we compared the trend of the urinary viral load values obtained at the different times of the follow up within each cohort, we did not find a statistically significant difference ($p>0.05$) (data not shown).

Results on plasma samples showed the presence of JCV in 6/18 and 3/25 CD patients in *cohort 1* and *cohort 2* respectively at t0. At t1 in *cohort 1*, the patients with viremia were 7/18 and 2/25 in *cohort 2*. At t2 and t3, we observed a decrease of the JCV positive plasma specimens up to 5/18 in *cohort 1* and up to 1/25 in *cohort 2* (Table 4). In particular, the number of patients with JCV viremia was significantly higher in *cohorts 1* than in *cohort 2* at t1, t2 and t3 ($p<0.05$), except for the time t0 ($p>0.05$) (Table 4). Comparing the plasma viral loads of both cohorts, we observed that viremia significantly increased in *cohort 1* respect to *cohort 2* at t1 ($p=0.05$), as shown in Table 4. In fact in *cohort* and in *cohort 2* the median viral load at t1 was 6.30 log GEq/mL and 4.56 log Geq/mL respectively (Table 4). Nevertheless, the trend of patients with JC viremia values was not statistically significant in each cohort during the follow-up ($p>0.05$) (data not shown).

Concerning JCV detection in the ileal and colon-rectal biopsies in *cohort 1* and *cohort 2*, results showed that at t0 JCV was found in 10/18 ileal specimens and in 8/18 colon–rectal biopsies of *cohort 1* and in 9/25 ileal specimens and in 4/25 colon–rectal biopsies of *cohort 2*. At t3, we found JCV in 7/18 ileal biopsies and in 4/18 colon–rectal biopsies of *cohort 1* and in 5/25 ileal samples and in 2/25 colon–rectal biopsies of the *cohort 2* (Table 5). However, the number of patients with JCV positive biopsies samples did not reveal a significant association in the two cohorts at t0 and t3 ($p>0.05$), except for the samples collected from colon–rectum at t0 ($p=0.04$) (Table 5). Comparing the JCV viral loads found in the biopsies of the two cohorts, we found only a significantly increasing in *cohort 1* respect to *cohort 2* in the ileal biopsies at t3 ($p=0.042$); in fact, in the

Table 3 JCV detection in the urine specimens and relative viral load in the cohort 1 and cohort 2 at different times of sampling (t0, t1, t2, and t3)

	Neg	Pos	Tot	p^c	Viral load, median (range), log GEq/mL	p^d
JCV Urine t0						
cohort 1[a]	12	6	18	0.70	7.35 (4.85–8.85)	0.193
cohort 2[b]	18	7	25		4.70 (2.98–9.62)	
JCV Urine t1						
cohort 1	8	10	18	0.31	6.12 (3.17–8.37)	0.496
cohort 2	15	10	25		5.07 (3.72–8.93)	
JCV Urine t2						
cohort 1	12	6	18	0.18	5.85 (4.30–8.15)	0.286
cohort 2	21	4	25		5.57 (3.92–5.88)	
JCV Urine t3						
cohort 1	12	6	18	0.09	7.47 (5.63–9.39)	0.039
cohort 2	22	3	25		5.36 (4.23–5.92)	

GEq genome equivalents

[a] Cohort of standard therapy nonresponders CD patients and then treated with infliximab

[b] Cohort of standard therapy responders CD patients

[c] By χ^2 test

[d] By Mann–Whitney *U* tests

Table 4 JCV detection in the plasma specimens and relative viral load in the cohort 1 and cohort 2 at different times of sampling (t0, t1, t2, and t3)

	Neg	Pos	Tot	p^c	Viral load, median (range), log GEq/mL	p^d
JCV Plasma t0						
cohort 1[a]	12	6	18	0.09	5.93 (4.06–7.58)	0.523
cohort 2[b]	22	3	25		5.20 (4.49–6.20)	
JCV Plasma t1						
cohort 1	11	7	18	0.01	6.30 (4.78–6.59)	0.050
cohort 2	23	2	25		4.56 (3.92–6.54)	
JCV Plasma t2						
cohort 1	13	5	18	0.03	4.08 (3.58–7.11)	0.770
cohort 2	24	1	25		5.18	
JCV Plasma t3						
cohort 1	13	5	18	0.03	4.63 (3.76–8.35)	0.587
cohort 2	24	1	25		4.13	

GEq genome equivalents

[a] Cohort of standard therapy non-responders CD patients and then treated with infliximab

[b] Cohort of standard therapy responders CD patients

[c] By χ^2 test

[d] By Mann–Whitney *U* tests

cohort 1, the median viral load at t3 was 5.85 log Geq/mL, whereas in the *cohort 2*, it was 3.97 log GEq/mL (Table 5). Finally, the trend of JC positive values in biopsies was not statistically significant in each cohort at t0 and at t3 ($p > 0.05$) (data not shown).

The study of the possible JCV NCCR rearrangements was carried out on JCV positive samples (51 for *cohort 2* and 80 for *cohort 1*), using a nested-PCR. Results evidenced that all the 51 JCV NCCR sequences (24 urine, 7 plasma, 14 ileal biopsies and 6 colon-rectal biopsies), obtained from *cohort 2*, showed the structural organization of the non-pathogenic archetype CY (Yogo et al. 1990) with random occurrence of a few irrelevant point mutations, that did not involve any cellular transcriptional factors binding sites (data not shown). The analysis of the 80 NCCR JCV sequences (28 urine, 23 plasma, 17 ileal biopsies and 12 colon-rectal biopsies), obtained from *cohort 1*, showed also the CY NCCR structural organization (Yogo et al. 1990) in 65/80 NCCR analyzed sequences, whereas the remaining 15/80 sequences, obtained exclusively from plasma and intestinal biopsies, evidenced the typical CY NCCR organization with a characteristic deletion of the box D and 2 recurrent nucleotide changes, a T to G transversion at position nucleotide number 37 (37-T to G) in the box A Spi-B binding site and a G to A transition at position nucleotide number 217 in the box F (217-G to A). In detail, the 4 NCCR sequences obtained from plasma presented both the nucleotide changes without the deletion of the box D (Table 6 and Fig. 1). Within the 7 NCCR sequences obtained from ileal biopsies, we found the two mentioned nucleotide changes in all the 7 NCCR sequences with the exception of 2 sequences that also showed the box D deletion (Table 6 and Fig. 1). Finally, among the 4 NCCR sequences obtained from colon-rectum biopsies, we found 3 sequences with only the two nucleotide changes, and 1 sequence that enclosed all the three mentioned characteristic rearrangements (Table 6 and Fig. 1). All these particular NCCR sequences were always found at t3 in 7 infliximab-treated pediatric CD patients of *cohort 1*. Finally, in order to exclude the possibility of a contamination, a VP1 genotype analysis was carried out on all positive samples of the 7 patients with these character-

Table 5 JCV detection in the ileum and colon–rectum biopsies and relative viral load in the cohort 1 and cohort 2 at different times of sampling (t0 and t3)

	Neg	Pos	Tot	p^c	Viral load, median (range), log GEq/10^5 cells	p^d
JCV Ileum t0						
cohort 1[a]	8	10	18	0.20	4.29 (3.54–6.57)	0.568
cohort 2[b]	16	9	25		5.73 (3.81–8.15)	
JCV Ileum t3						
cohort 1	11	7	18	0.17	5.85 (4.34–8.48)	0.042
cohort 2	20	5	25		3.97 (3.45–6.81)	
JCV Colon–rectum t0						
cohort 1	10	8	18	0.045	3.66 (3.10–4.67)	0.089
cohort 2	21	4	25		.38 (3.30–6.71)	
JCV Colon–rectum t3						
cohort 1	14	4	18	0.18	4.85 (3.52–7.08)	0.355
cohort 2	23	2	25		4.49 (3.38–4.78)	

GEq genome equivalents

[a] Cohort of standard therapy nonresponders CD patients and then treated with infliximab

[b] Cohort of standard therapy responders CD patients

[c] By χ^2 test

[d] By Mann–Whitney *U* tests

Table 6 Characteristics of NCCR sequences found in the positive samples within the cohort 1

Samples	Positive samples	CY archetype[a] (%)	Characteristics of rearranged NCCR sequences (%)	
			37 T to G[b] and 217 G to A[c]	37 T to G[b], 217 G to A[c] and Box D deletion
Urine	28	28 (100%)	0	0
Plasma	23	19 (83%)	4 (17%)	0
Ileum	17	10 (59%)	5 (29%)	2 (12%)
Colon–rectum	12	8 (67%)	3 (25%)	1 (8%)
Total (%)	80	37 (81%)	12 (15%)	3 (4%)

[a] JCV CY archetype (Yogo et al. 1990) with random occurrence of a few irrelevant point mutations

[b] T to G transversion at position nucleotide number 37 in the box A within Spi-B binding site

[c] G to A transition at position nucleotide number 217 in the box F

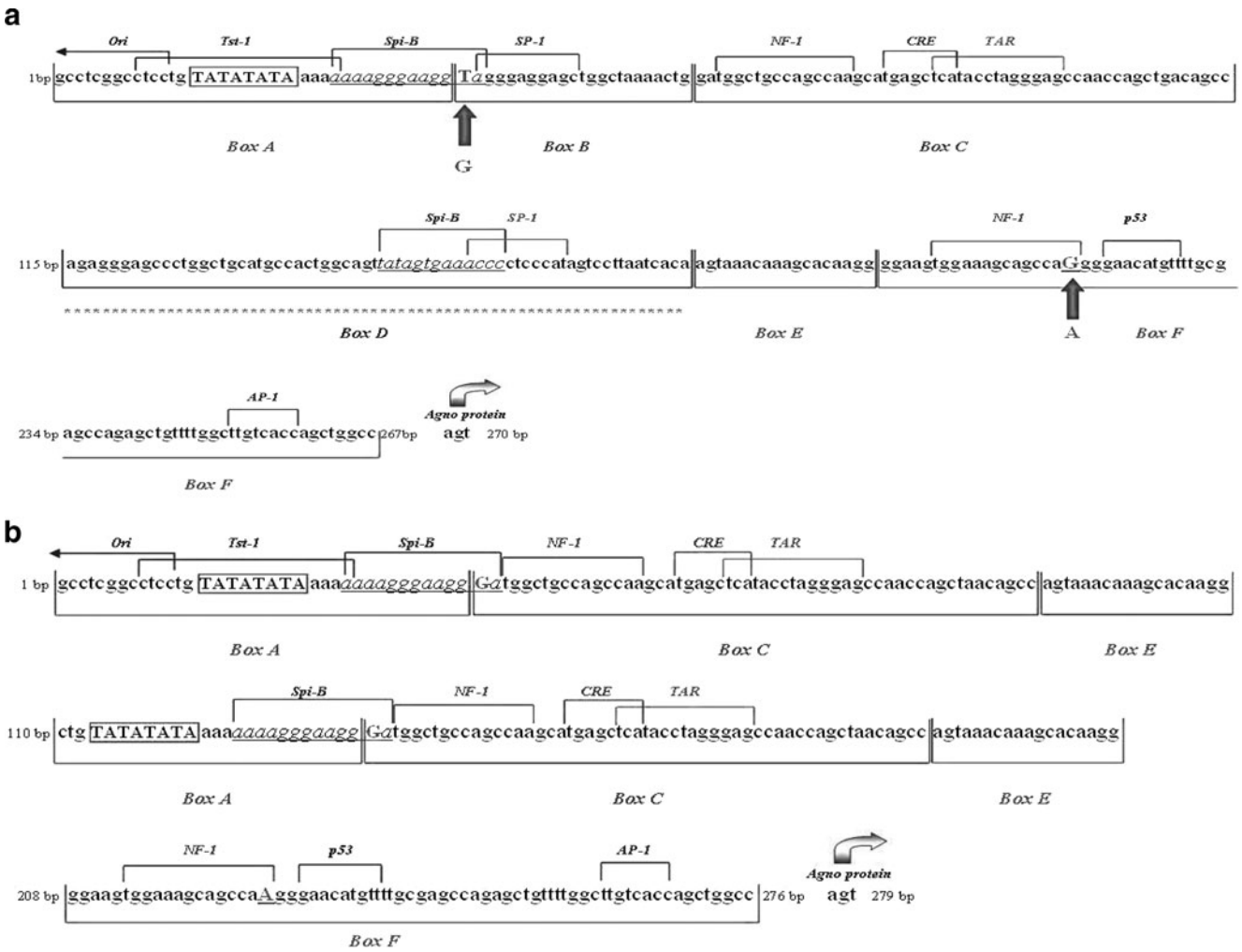

Fig. 1 Rearranged CY NCCR structure found in our samples (**a**) and PML-associated variant Mad-1 NCCR structure (**b**). The nucleotide sequences are shown from the origin of DNA replication (*Ori*) to the start site (AGT) of the late leader protein, *Agno protein* (**a** and **b**). In **a**, the nucleotide numbering is based on the archetype sequence of Japanese strain CY (Yogo et al. 1990). Regions from Box A to Box F are shown below the sequences (**a** and **b**) (Ault and Stoner 1993). TATA sequence, in *capital letters*, is marked with a *box*, and several transcriptional factor binding sites are indicated *above the sequences*: in particular, the Spi-B binding sites in both sequences are in *italic underlined* (**a** and **b**). The sequence in **a** were found in plasma specimens, in ileal and colon–rectal biopsies collected at t3 from seven patients treated with infliximab (*cohort 1*) that showed a PCDAI >40 at the moment of the enrolment. The two nucleotide changes (hot spot mutations) are written in *bold capital letters*, *underlined*, and indicated with *an arrow*. The deletion of the Box D is indicated with *asterisks*. The nucleotides found in our sequences are located *under the arrow*, and they are typical of the JCV NCCR structure of PML-associated variant Mad-1, as shown in **b** (Frisque et al. 1984)

istic rearrangements. Results showed the presence of genotype 1A in 5 patients and the presence of genotype 1B in 2 patients (data not shown).

Discussion

The recent introduction of mAbs in CD management, such as infliximab (Remicade, Centocor Ortho Biotech), has been associated with the development of serious infections. JCV reactivation in CD patients after mAbs therapy and its association with PML has been reported after using natalizumab (Major 2010; Marshall and Major 2010). Since the number of patients undergoing immunomodulatory therapies that develop PML continues to rise (Major 2009), the understanding of JCV reactivation is increasingly important. Moreover, the association between JCV infection and chronic idiopathic intestinal pseudo-obstruction (CIIP) leads to the hypothesis that this neurotropic virus may undergo activation from a latent state in the gut to an active lytic infection, with serious clinical consequences in affected patients (Selgrad et al. 2009). In fact, under normal conditions, T cell activation occurs in the setting of an antigen presented by antigen presenting cells (APCs). In presence of interleukin IL-12 and IL-18, secreted by APCs, the T cells are programmed to produce pro-inflammatory cytokines, such as interferon (IFN)-γ, TNF-α, and IL-2. Both IL-12 and IL-18 are found at increased levels in the intestinal mucosa of CD patients, and TNF-α is shown to be an important mediator of the intestinal inflammation because overall enhances recruitment of other inflammatory cells. Therefore, the TNF-α blocking by infliximab results in a decreased expression of IL-1, IL-6, IFN-γ, and other pro-inflammatory cytokines (Bellizzi et al. 2010). Since the use of infliximab in CD patients blocks the TNF-α and interferes with the recruitment of lymphocytes causing a decreasing of IFN-γ levels, involved in the anti-viral state control, JCV could leave its latent state within the enteric glial cells (Selgrad et al. 2009) and could infect the intestinal epithelium. Moreover, JCV could also restart its lytic cycle in other cell types, including the CD34+ haematopoietic precursors, the B cells present in bone marrow and in circulation (Tan et al. 2009) and the renal tubular epithelial cells (Randhawa et al. 2001). Considering these assumptions, the first aim of our study was to evaluate by Q-PCR the possible role of infliximab in JCV reactivation collecting urine, plasma and intestinal biopsies from two cohort of pediatric CD patients treated or not with this mAb at different times. The second part of our research has been directed to the study of JCV NCCR sequences.

The Q-PCR analysis highlighted a significant increase of the total number of JCV positive specimens in *cohort 1* (80/216) compared to *cohort 2* (51/300). These results were supported by the diagnosis of a more severe CD (PCDAI> 30) affecting the patients treated with infliximab, which alters the anti-viral state in the intestinal compartments.

The results of viruria showed the same trend of the number of patients in both cohorts, with an increase 4 months (t1) after the beginning of the therapies and a decrease at t2 and t3, although the small size of the studied populations did not provide statistically significant results. These data could be explained by the highest effectiveness reached by infliximab and standard therapy at t1, which could interfere with the mild balance between anti-inflammatory drug benefit and immune surveillance. The decrease of the number of patients observed at t2 and t3 within the two cohorts could be the result of the improvement of CD after 8 and 12 months of therapy, especially for the patients of *cohort 2*. Concerning the urinary viral loads, we observed at t3 a significant increase of the viruria in the patients treated with infliximab compared to the patients receiving a standard therapy (7.47 log GEq/mL versus 5.36 log GEq/mL), whereas at t1 and t2, the urinary viral loads maintained median values between 5 and 6 log GEq/mL in both cohorts. This result puts in highlight a higher viral replication in the renal tubular epithelial cells with a shedding of the virus in the urine after 1 year of infliximab treatment.

Concerning the plasma samples, we recorded a similar number of patients in *cohort 1* with viremia during all the follow-up, whereas in *cohort 2*, we observed a strongly opposite trend, with a lower number of patients with viremia at t0 (3/25), that decreased at the subsequent times (t1, t2, t3) of the follow-up. The significant higher number of patients with viremia in *cohort 1* respect to *cohort 2* at t1, t2, and t3 ($p<0.05$) could be explained with a more severe clinical status of CD patients in *cohort 1* and their treatment with infliximab. Moreover, the administration of infliximab could be considered a trigger for JCV reactivation from its latent state within a variety of cell types of the bloodstream, including CD34+ hematopoietic precursors and B cells, by which JCV could reach the brain (Tan et al. 2009). On the basis of these results and considering that the upregulation of the genes involved in B-cell differentiation, including the lymphotropic transcriptional activator Spi-B, has been observed in response to treatment with natalizumab (Lindberg et al. 2008; Major 2010), we could also hypothesize that the infliximab therapy may cause mobilization of CD34+ hematopoietic precursors and B cells that have the potential to harbor latent JCV infection. This hypothesis could also be supported by the median of viremia that reached the value of 6.30 log GEq/mL in the patients treated with infliximab at t1, when the drug achieved its highest effectiveness.

Finally, regarding all the ileal biopsies examined, we observed that the number of patients with JCV positive

biopsies decreased in both cohorts 12 months after the treatments onset (t3), with a no significant statistical association, in spite of the number of patients with JCV positive colon–rectal biopsies was significantly higher in *cohort 1* than *cohort 2* at t0 ($p<0.05$). Nevertheless, the viral load found in the biopsies from ileum increased significantly in *cohort 1* ($p<0.05$) respect to *cohort 2* at t3, whereas no statistical significant correlation was found between the JCV viral loads detected in colon-rectal biopsies. Although the meaning of these results was related to the small size of the studied population, we can hypothesize that JCV may undergo reactivation in the gut with a spreading of virions in the bloodstream, according to Selgrad and collegues who speculated that this neurotropic virus may switch its latent state in the enteric neurons on an active lytic infection, with serious clinical consequences in CIIP patients (Selgrad et al. 2009).

The analysis of the JCV NCCR sequences showed that all the 51 sequences obtained from *cohort 2*, had the structural organization of the non-pathogenic archetype CY (Yogo et al. 1990), with random occurrence of a few irrelevant point mutations, that did not involve any cellular transcriptional factors binding sites, and with none of the deletions and duplications typical for isolates of PML strains. This finding is in agreement with the gradual but effective CD remission in the pediatric patients of *cohort 2*, confirmed by a PCDAI value <10 at t3 (data not shown). On the other hand, the analysis of the 80 JCV NCCR sequences obtained from *cohort 1* showed a CY archetype NCCR structure in 65/80 analyzed sequences (Yogo et al. 1990), whereas the remaining 15/80 sequences evidenced rearranged CY NCCR with a characteristic deletion of the box D and with two nucleotide changes, the 37-T to G transversion in the box A Spi-B binding site and the 217-G to A transition in the box F. These NCCR sequences were always found at t3 in plasma samples and intestinal biopsies of 7 infliximab-treated pediatric CD patients of *cohort 1*. Interestingly, all these patients presented at t0, t1, and t2 a CY archetype NCCR structural organization and they showed a PCDAI value >40 at t0. Moreover, all the rearrangements detected at t3 are completely random, as revealed by the VP1 genotype analysis performed on these 7 patients. In fact, the VP1 genotype analysis showed the presence of genotype 1A in 5 patients and the presence of genotype 1B in 2 patients (data not shown), in agreement with the most common genotypes detected in the European population as described by some Authors (Agostini et al. 2001; Mischitelli et al. 2010). The finding of these rearranged JCV NCCR sequences at t3 could be explained by the interference of infliximab in the regulation of the fine balance between anti-inflammatory drug benefit and immune surveillance, also taking into account the timing of administration of this mAb during the maintenance therapeutic program. Moreover, the infliximab activity results in a decreased expression of pro-inflammatory cytokines such as IFN-γ that is important to control the anti-viral state of latent viruses. In fact, we found the rearranged JCV NCCR sequences after 1 year of infliximab therapy, and therefore, we could suppose that they depend on the severity of the CD illness and on the immunological unbalanced state induced by mAb therapy. According to these data, we observed that the 37-T to G transversion in the box A shifted the typical CY archetype Spi-B binding site (5′-AAAAGGGAAGGTA-3′) to the characteristic one of the PML-associated variant Mad-1 (5′-AAAAGGGAAGGGA-3′). The finding of this nucleotide change could enhance JCV replication because it has been demonstrated that, the Spi-B-binding sites that actively bind the Spi-B protein expressed in JCV susceptible cell types, are present in Mad-1 NCCR sequences, but not in the non-pathogenic archetype CY (Marshall et al. 2010). For this reason, understanding the role of Spi-B during JCV latency and reactivation is increasingly important, considering also the hypothesis that JCV could use CD34+ hematopoietic precursors and B cells, present in the bloodstream, to reach the brain (Garrett-Sinha et al. 1999; Su et al. 1996). Moreover the finding of the shift of the typical CY archetype Spi-B binding site in the characteristic one of the PML-associated variant Mad-1, in association with the box D deletion in two patients, let us conclude that JCV is attempting to enhance its virulence, as a consequence of immunological alterations of the host. Furthermore, the finding of the 217-G to A transition in the box F is completely in agreement with other data present in literature; in fact, this nucleotide change seems to be a common feature of the European strains as just described by Agostini and colleagues (Agostini et al. 1996), and it has been also observed by Yogo and colleagues in several strains, including Mad-1, in the course of an infection by JCV in the permissive cells (Yogo et al. 1990). Therefore, we can highlight that our rearranged NCCR sequences could be considered a marker of the JCV virulence during the mAb treatment, although none of our examined patients developed PML, and further studies on a larger cohort of pediatric CD patients should be performed. In conclusion, since the number of patients, affected by autoimmune disease and treated with mAbs, continues to rise (Major 2009), we could take into account that the follow-up of JCV DNA detection and the subsequent JCV NCCR sequences analysis in this class of patients may be important in order to reduce the risk of PML onset.

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