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A new protoparvovirus in human fecal samples and cutaneous T cell lymphomas (mycosis fungoides)

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We genetically characterized seven nearly complete genomes in the protoparvovirus genus from the feces of children with diarrhea. The viruses, provisionally named cutaviruses (CutaV), varied by 1–6% nucleotides and shared 76% and 82% amino acid identity with the NS1 and VP1 of human bufa-viruses, their closest relatives. Using PCR, cutavirus DNA was found in 1.6% (4/245) and 1% (1/100) of diarrhea samples from Brazil and Botswana respectively. In silico analysis of pre-existing metagenomics datasets then revealed closely related parvovirus genomes in skin biopsies from patients with epi-dermotropic cutaneous T-cell lymphoma (CTCL or mycosis fungoides). PCR of skin biopsies yielded cu-tavirus DNA in 4/17 CTCL, 0/10 skin carcinoma, and 0/21 normal or noncancerous skin biopsies. In situ hybridization of CTCL skin biopsies detected viral genome within rare individual cells in regions of neoplastic infiltrations. The influence of cutavirus infection on human enteric functions and possible oncolytic role in CTCL progression remain to be determined.

1. Introduction

Parvoviruses are small non-enveloped viruses with single-stranded linear DNA genomes of approximately 5-kb. These viruses can infect mammalian hosts resulting in asymptomatic to highly pathogenic infections (Berns and Parrish, 2013). The Primate protoparvovirus 1 species within the genus Protoparvovirus is currently comprised of human bufaviruses (BuVs), while rhesus macaque BuV, shrew BuV, and bat BuV may belong to other Protoparvovirus species based on the NS1 genetic distance criteria proposed by the International Committee on Taxonomy of Viruses (Cotmore et al., 2014; Handley et al., 2012; Sasaki et al., 2016; Sasaki et al., 2015). Human BuVs can be further divided into three genotypes based on their structural protein 1 (VP1) (Phan et al., 2012; Yahiro et al., 2014).

Bufaviruses in human feces have been reported in a number of countries. BuV1 and 2 genomes were initially described in feces of children with unexplained diarrhea from Burkina Faso (4% or 4/98) and feces from a Tunisian child with non-poliovirus acute flaccid paralysis (1.5% or 1/68) (Phan et al., 2012). BuV3 was then detected in feces collected from Bhutanese children with diarrhea (0.8% or 3/393) (Yahiro et al., 2014). BuV1 was also detected in fecal specimens from diarrheal patients in Finland (1.1% or 7/629) (Vaisanen et al., 2014)

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and BuV3 sequences were reported in an idiopathic human diarrheal sample from the Netherlands (7% or 1/14) (Smits et al., 2014). BuV1 DNA was detected by PCR in diarrheal samples from Thailand (0.27% or 4/1495) but not in 726 non-diarrheal feces (Chieochansin et al., 2015). A Turkish study detected BuV3 DNA in diarrheal samples (1.4% or 8/583) but not in feces from 148 healthy children (Altay et al., 2015). BuVs 1 and 3 were reported from pediatric and adult diarrheal patients in China (0.5% or 9/1877) (Huang et al., 2015). BuVs are therefore widely geographically distributed and detectable at low frequency in human diarrheal samples although a statistically significant association with diarrhea remains to be shown and may require larger case control studies in countries with high prevalence.

The first BuV-related sequences identified in non-human primates were obtained from fecal and serum samples collected from simian immunodeficiency virus-infected rhesus monkeys (Macaca mulatta) in a U.S. primate center (Handley et al., 2012). The simian parvovirus WUHARW shared an amino acid (aa)-identity of 57% to NS1 and 69% to VP1 of human BuV (Handley et al., 2012). Other BuV-related protoparvovirus genomes were also reported in bat feces from Hungary (Miniopterus schreibersii) (Kemenesi et al., 2015), and China (Myotis ricketti and Myotis formosus) (Wu et al., 2015), in bat feces and spleen tissues from Indonesia (Pteropus vampyrus) (Sasaki et al., 2016), in feces, spleen and liver tissues of wild shrews from the Crocidura genus, yellow baboons (Papio cynocephalus), and a chacma baboon (Papio ursinus) from Zambia (Sasaki et al., 2015), in wild rat feces from China (Yang et al., 2016), and pig feces from Hungary and Australia (Hargitai et al., 2016; Liu et al., 2016).

In this study, we describe the genome of a new protoparvovirus detected in both human diarrhea samples and epidermotropic cutaneous T-cell lymphoma (mycosis fungoides) (Willemze et al., 2005) skin biopsies, further widening the genetic diversity of human associated protoparvoviruses and expanding their tropism to non-enteric cells.

2. Materials and methods

2.1. Clinical samples and viral metagenomics

Pools of five fecal specimens were assembled and fecal supernatants filtered through a 0.45-mm filter (Millipore) to remove bacterium-sized particles. The filtrates were incubated with DNase and RNase to digest unprotected nucleic acids (Victoria et al., 2009). The unprotected nucleic acids were then extracted using a Maxwell 16 automated extractor (Promega). DNA was synthesized by random RT-PCR, followed by the use of the Nextera™ XT Sample Preparation Kit (Illumina) to construct DNA libraries (Phan et al., 2015). Two Illumina MiSeq runs of 250 bases paired-ends were made, yielding an average of 878 thousand sequence reads from each pool of fecal specimens. After removal of duplicate reads followed by de novo assembly, viral sequences were identified through translated protein sequence similarity search (BLASTx) to annotated viral proteins available in GenBank's viral RefSeq database.

2.2. Genome acquisition of a new parvovirus and genetic analysis

The nearly complete genome was determined by filling genome gaps by PCRs with primers designed from initial BuV-like sequences, and amplifying its extremities using RACE. Amplicons were directly sequenced by primer walking. Sequence alignment was performed using CLUSTAL X (version 2.0.3), and sequence identities were measured using BioEdit. Phylogenetic analyses were based on the translated protein sequences. Phylogenetic trees with 100 bootstrap resamples of the alignment data sets were constructed using the maximum likelihood method. Bootstrap values (based on 100 replicates) for each node are given if ≥70%. The generated phylogenetic trees were visualized using the program MEGA version 6 (Tamura et al., 2013). Simplot was used to calculate nucleotide similarities along the length of parvoviral genomes (Lole et al., 1999).

2.3. PCR assays for a new parvovirus

Two sets of nested-PCR primers targeting VP1 were designed to screen nucleic acids extracted from feces and skin biopsies using a Maxwell 16 automated extractor (Promega) and MyTaq Extract-PCR Kit (Bioline), respectively. Primer set A was CutaV-F1 (position primers based on GenBank KT868811) (5′-^{3361}CAA ACT ACC AAC TTA CTG CTA CCA^{3384}-3′) and CutaV-R1 (5′-^{3834}GTT AGT CTG GTT CCT TCA GTT GTT G^{3858}-3′) were used for the first round of PCR, and primers CutaV-F2 (5′-^{3397}GAA TAC AAT AGA CAT AAA CCA AGC AGA C^{3424}-3′) and CutaV-R2 (5′-^{3801}TGC TTG TGA AAA TGA ACT GCC TG^{3823} 3′) for the second round of PCR, generating a 427-bp amplicon. The PCR conditions were: 95 °C for 5 min, 35 cycles 95 °C for 30 s, 50 °C (for the first or second round) for 30 s and 72 °C for 1 min, a final extension at 72 °C for 10 min

For primer set B we used CutaV-F3 (5′-^{3225}GA GAA ACA GAA ACA ACC AAC TAC AAC AA^{3252}-3′) and CutaV-R3 (5′- ^{3682}TTG TTT CTG TCT CCC CAT TGC^{3702}-3′) in the first round of PCR, and primers CutaV-F4 (5′-^{3264}CAC TCC TAC AAA TAG CAC AAG ACA ACA G^{3291}-3′) and CutaV-R4 (5′-^{3590}CTG CCT GTG TGT CTT TGT GAT TG^{3612}-3′) for the second round of PCR, generating a 349-bp amplicon. The PCR conditions were: 95 °C for 5 min, 35 cycles 95 °C for 30 s, 50 °C for the first or 51 °C for the second round) for 30 s and 72 °C for 1 min, a final extension at 72 °C for 10 min.

2.4. In situ hybridization

Colorimetric ISH was performed manually on 5 mm sections of formalin-fixed, paraffin-embedded biopsy tissue on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) using the RNAscope 2.0 BROWN assay kit (Advanced Cell Diagnostics, Inc., Hayward, CA). Briefly sections were treated with heat and protease before hybridization with the probe cocktail which includes 17 pairs of probes within a target region (25-968) of the virus. Cuta-V probe is publicly available for purchase through Advanced Cell Diagnostics (V-Cuta). Development of hybridization was visualized with 3,3′-diaminobenzidine (Advanced Cell Diagnostics, Inc., Hayward, CA). Slides were counterstained with hematoxylin (2 min, 50% hematoxylin) and mounted with xylene-based SHUR/Mount (Triangle Biomedical Sciences, Durham, NC). Assays were done in duplicate or triplicate and two probes were used as negative controls for the study; a scrambled GC content matched probe and a probe to the bacterial gene, dapB (diaminopimelate B) were performed in serial sections in each case. A positive CutaV test result was defined as punctate staining.

3. Results

3.1. Genetic characterization of a new parvovirus species

Using metagenomics we performed a virome analysis of the feces of 255 Brazilian children with unexplained diarrhea that had previously tested negative for rotavirus, and adenovirus (da Cruz et al., 2014). Two pools of five samples were found to have bufavirus related sequence reads. The individual fecal specimens (BR-337 and -372) within the two pools containing these sequences were then identified using PCR with primer set A (Material and Methods). A nearly complete 4456-bp parvovirus genome was then acquired by filling gap using PCR and Sanger sequencing and the virus was named cutavirus (CutaV-BR-337) (GenBank

KT868811 for cutaneous T-cell lymphoma associated parvovirus). The partial genome encoded complete open reading frames (ORF) of NS1 (659-aa), VP1 (707-aa), and a middle ORF of unknown function (Fig. 1(A)). Since the genome of CutaV-BR-337 was most closely related to that of human bufaviruses it was aligned with these genomes to identify theoretical splicing signals for the expression of VP1. The NS1 of CutaV-BR-377 (650-aa) was shorter than those of human BuV1–3 (671aa -673-aa), showing a large 11 aa deletion near the carboxyl end (Fig. S1). The NS1 contains the helicase with characteristic NTP-binding domains A [^{396}TILLCGPASTGKSL^{409}], B [^{441}LIWIEE^{446}] and C [^{494}VIVGT^{498}] (Gorbalenya et al., 1990) (Fig. S2). Similar to other members in the BuV clade, the VP1 contained phospholipase A2 (PLA2) and glycine-rich regions (G-rich) (Phan et al., 2012; Sasaki et al., 2015; Yahiro et al., 2014). The pair-wise aa-sequence analysis demonstrated that cutavirus NS1 and VP1 showed closest identity of 76% and 82% to those of human BuV2, respectively. The middle ORF encoding a theoretical protein of unknown function (109-111 aa long) of unknown function was shorter than those of human BuV1-3 (130-aa), sharing a low identity of 45% (Phan et al., 2012; Yahiro et al., 2014). In addition to three major ORFs described above, the NCBI ORF finder revealed a small ATG initiated ORF (89–92 aa long) located over the VP2 coding region also showing no similarity to known proteins by BLASTp, which was conserved in all seven nearly complete cutavirus sequences in this study. A late non-structural protein named SAT (small alternatively translated) was shown to be expressed from this conserved ORF which when mutated in porcine parvovirus showed a slow spreading phenotype in vitro (Zádori et al., 2005). ORFs over the same location were detected in all bufaviruses (82-103 aa) and in other protoparvoviruses (53-74 aa). Phylogenetic analyses of the complete NS1 and VP1 of cutavirus proteins were performed (Fig. 1(B) showing a common root with human bufaviruses). According to the International Committee on Taxonomy of Viruses, member of the same parvovirus species share more than 85% of aa-sequence identity in the NS1 ORF (Cotmore et al., 2014). The 76% NS1 identity between human bufaviruses and cutaviruses indicate that cutaviruses may be classified as members of a distinct protoparvovirus species.

3.2. Detection of additional CutaVs in other human diarrhea fecal specimens

A nested PCR assay with primer set A was used to investigate the prevalence of CutaV DNA in 345 diarrhea feces (245 from Brazil and 100 from Botswana). Beyond the two cutaviruses initially detected by deep sequencing two additional Brazilian feces (BR-283 and -450) were also PCR positive, yielding a prevalence of 1.6% (4/245) in this population. Only one of one hundred (1%) Botswana feces tested (BO-46) was PCR positive. The genomes of Brazilian CutaV-BR-283 (4258-bp; GenBank KT868810), BR-372 (3985-bp; GenBank KT868809), BR-450 (3983-bp; GenBank KT868812), and BO-46 from Botswana feces (4148-bp; GenBank KT868813) were then partially sequenced. While the complete VP1 and middle ORFs of these CutaV strains could be amplified and sequenced, 256–473 nucleotides encoding the N-terminal ends of NS1 could not be amplified from these four samples possibly due to divergence in the 5′ region preventing primer annealing. Pair-wise sequence analysis of the partial genomes demonstrated 94–97% nucleotide identity except for BR-337 and BO-46 which shared 99% identity differing only at 21 nucleotide positions.

3.3. Detection of other viruses in CutaV containing diarrhea fecal specimens

In order to search for other enteric pathogens in the CutaV DNA positive diarrhea specimens, deep sequencing was similarly performed on individual specimens. The five fecal specimens containing CutaV (BR-283, -337, -372, -450 and BO-46) were therefore processed separately (rather than in pools) for viral metagenomics (Table 1). The specimen BR-283 also contained rotavirus A (0.6%) and adeno-associated virus (AAV) (0.05%). The specimen BR-337 also contained human astrovirus 1 (5.7%) and adenovirus (0.02%). The specimen BR-450 contained rotavirus A (7.8%) and anellovirus (0.05%). The specimen BO-46 contained picobirnavirus (0.12%) and anellovirus (0.013%). No other eukaryotic viruses were identified in the specimen BR-372.

3.4. Detection of CutaV DNA in human skin biopsies by digital screening and PCR

In order to search for nucleotide sequences closely related to the cutavirus genome we used MegaBlast to query our sequencing database consisting of multiple Illumina Miseq, Hiseq and 454 Roche viral metagenomics datasets previously generated in our laboratory from humans, animals, and environmental samples (Phan et al., 2015). Nucleotide sequences closely related to CutaV were only detected in one dataset generated three years earlier from a pool of four skin biopsies from French patients with mycosis fungoides (2 samples) and parapsoriasis (2 samples). Total nucleic acids of these four skin tissues were individually extracted and tested for CutaV DNA by PCR with primer set A. The two French mycosis fungoides skin tissues (FR-D and FR-F) were found to be PCR positive. PCR screening with primer set A was then performed on an additional 12 skin biopsies from French patients with skin carcinoma (10 samples) or parapsoriasis (2 samples) all of which were CutaV DNA negative. The nearly complete CutaV genomes (missing UTRs) from mycosis fungoides skin biopsies FR-D (4452-bp; GenBank KT868814) and FR-F (4452-bp; GenBank KT868815) were then acquired. Genome alignment showed 96% nucleotide identity between the French skin biopsies-associated CutaV sequences. While the amino acid identity among feces and skin biopsies-associated CutaV NS1 or VP1 proteins were 95–99%, the aa-identity between their middle ORF proteins ranged from 69 to 97% and was therefore the most variable ORF with its length differing by up to two codons. The Simplot nucleotide analysis of the three longest cutavirus genomes (with complete NS1, middle and VP1 ORFs) showed the middle ORF and the 5′ of the NS1 ORF to be the most variable regions (Fig. 1(C)).

In order to further investigate the relationship between CutaV DNA detection and mycosis fungoides, the nPCR assays with primer sets A and B were used to test for CutaV DNA in another 15 French mycosis fungoides skin biopsies and a group of seventeen samples including four eczema, six parapsoriasis, four eczematoid dermatitis, and three healthy skin biopsies. Two additional mycosis fungoides skin tissues (MF-1 and -6) were found positive for CutaV DNA by the nPCR assays with primer sets A and B. A total of 4/17 CTCL samples were therefore PCR positive while all 31 non-CTCL biopsies in the control groups were PCR negative (Fisher's exact test p¼0.022). The 531-bp VP1 fragments of the two French MF-1 and -6 CutaV genomes differed by 10 nucleotides (1.9%). The VP1 fragments of the MF-1 and -6 CutaV genomes showed 10–23 nucleotide mutations relative to those of other CutaV strains found in French skin tissues (FR-D and -F), Brazilian (BR-283, -337, -372, -450) and Botswana (BO-46) diarrhea samples.

3.5. Detection of CutaV nucleic acids using in situ hybridization of CTCL skin biopsies

Paraffin embedded, punch biopsy skin samples from 9 CTCL (four PCR positive, 2 PCR negative, and 3 PCR untested) and

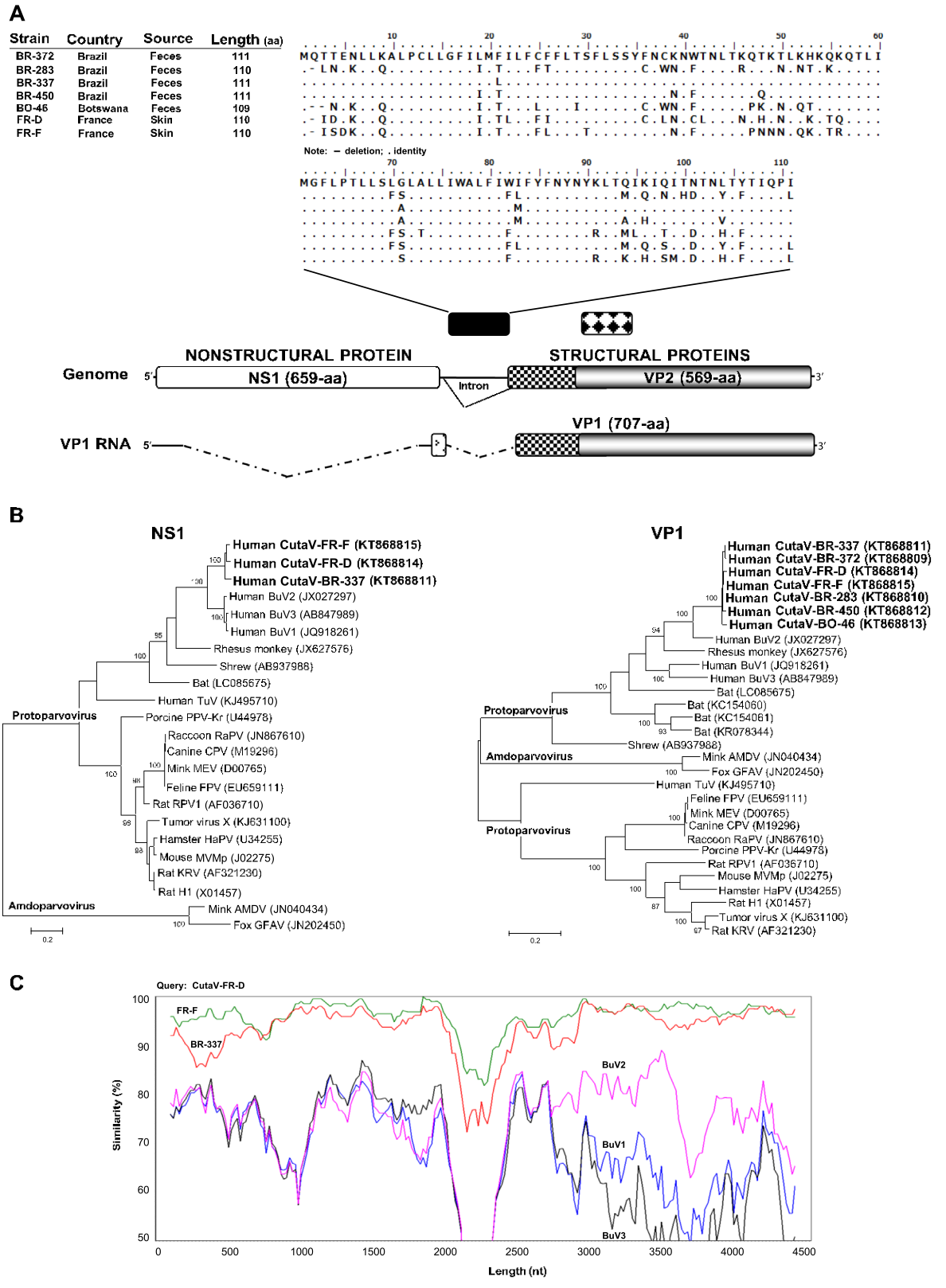

Fig. 1. New cutavirus genome and phylogeny. (A) Organization of the CutaV genome. Theoretical splicing for expression of VP1 and amino acid alignment of the middle ORF proteins were shown. (B) Phylogenetic trees generated with NS1 and VP1 of cutaviruses and other members of the genus Protoparvovirus. The scale indicated amino acid substitutions per position. Bootstrap values (based on 100 replicates) for each node are given if 4 70. (C) Pairwise sliding window of percent nucleotide similarity of the CutaV-FR-D aligned with other cutaviruses and human BuV1-3.

Table 1
Other eukaryotic viruses in five cutavirus positive fecal specimens using a metagenomics approach.

Specimens	Total of reads	Anellovirus	Picobirnavirus	Rotavirus A	Astrovirus 1	Adenovirus	AAV
BR-283	210,634			1337			107
BR-337	28,856				1684	6	
BR-372	55,694						
BR-450	25,018	14		1957			
BO-46	356,158	46	448				

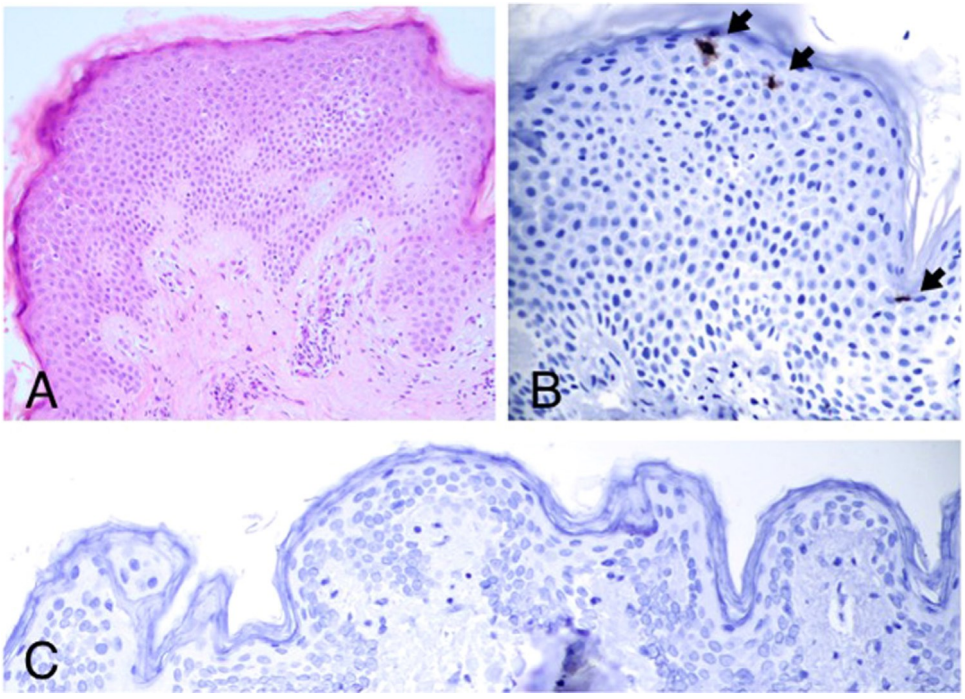

Fig. 2. In situ hybridization of skin biopsy of mycosis fungoides. (A) There are perivascular and scattered lymphocytes within the dermis, and epidermotropic T cells of Mycosis Fungoides penetrate the epithelium individually and in small clusters (Pautrier microabscesses). (B) In the upper strata of the skin sample, cutaviral DNA is detectable within 3 cells in the section. The vast majority of neoplastic and epithelial cells are negative. Arrows indicate ISH cutaviral probe signal. (C) Normal skin.

3 healthy subjects (2 PCR negative and 1 PCR untested) were hybridized with cutavirus DNA probes using the RNAscope method. The pathologist reading the ISH slides was blinded to the diagnoses. Rare but strongly positive cells were detected in 2/4 CTCL tissues positive by PCR but in none of the other 5 CTCL and 3 healthy subjects tested (Fig. 2). In 5 mm sections taken in sequential series, positive cells were reproducibly (3) detected in the same region of the CTCL biopsies (data not shown). Controls including normal skin, inflamed skin, and scrambled probe (nucleotide matched) on CTCL skin were negative (Fig. 2).

The positive signal, in both cases, was within individual cells in regions within the upper spinous layer directly adjacent regions where neoplastic T cells infiltrated the epithelium. Because the epithelial layer was disrupted in these regions, it was unclear whether the signal was present within T cell lymphocytes of the infiltrate of mycosis fungoides or within epithelial cells.

4. Discussion

To date six different types of parvoviruses (namely AAV, B19, PARV4, bocaviruses, tusavirus and bufaviruses) have been identified in human samples (Brown, 2010; Phan et al., 2014, 2012). We genetically characterized a new parvovirus detected in both human feces from patients with diarrhea and skin biopsies from human mycosis fungoides. By deep sequencing five CutaV containing diarrhea samples, we also detected recognized enteric pathogens (rotavirus, adenovirus and astrovirus 1) in three samples. Anelloviruses were also found in two diarrhea feces. Anellovirus infections are common in humans, and like AAV are generally considered to be commensal infections (Brown, 2010; Okamoto, 2009; Spandole et al., 2015; Zinn and Vandenberghe, 2014). Another diarrhea sample contained picobirnavirus sequences; however, these recently described common and highly diverse viruses have also not yet been strongly associated with human diarrhea (Ganesh et al., 2012; Malik et al., 2014). No viral sequences other than CutaV were detected in the fifth diarrhea sample. A potential pathogenic role of CutaV with or without other enteric infections should therefore be considered.

Parvoviruses are generally considered lytic rather than oncogenic viruses. Nonetheless B19 parvovirus has been reported in thyroid carcinomas (Adamson et al., 2011; Wang et al., 2008) and lymphomas (Polcz et al., 2013). Human bocavirus DNA was also detected in formalin-fixed, paraffin-embedded tissues from lung and colorectal cancers (Schildgen et al., 2013) and AAV were recently found integrated upstream of oncogenes in hepatocellular carcinoma (Nault et al., 2015). Because the two mycosis fungoides skin biopsies initially analyzed by metagenomics were processed to enrich viral particle-associated nucleic acids, the detection of CutaV DNA may reflect ongoing replication or deposition of viral particles in skin rather than an oncogenic role. The low level of cells positive by in situ hybridization also argues against an

oncogenic role as viral expression would be expected in most transformed T lymphocytes in the infiltrate of mycosis fungoides. Parvovirus B19 DNA is also detectable in skin biopsies years to decades past initial infection (Norja et al., 2006) indicating that CutaV DNA detection in skin biopsies may reflect a similar phenomenon of low level replication and/or occasional re-activation as seen for B19 (Parsyan and Candotti, 2007).

Precedents abound for oncolytic infections of tumor cells by protoparvoviruses. The rat protoparvovirus H-1 efficiently kills different cancer cell lines, including human melanoma-derived cells, without affecting normal cells (Rommelaere et al., 2010) and is being tested as therapeutic agent against glioblastoma multiforme (Marchini et al., 2015). Tumor virus X, grown from a permanent amnion cell line, with 93% nucleotide similarity to Kilham rat virus and 81% similarity to rat H1 parvoviruses also has strong oncolytic properties against human melanoma cells (Vollmers et al., 2014; Vollmers and Tattersall, 2013). Rat protoparvoviruses have been shown to be lymphocytotropic (Gaertner et al., 1993; McKisic et al., 1993; McKisic et al., 1995). In a potentially analogous situation a chronic systemic parvovirus infection was reported in a slow loris (Nycticebus coucang) with a histiocytic sarcoma (Canuti et al., 2014). Whether the detection of cutaviruses in mycosis fungoides skin biopsies indicates preferential replication in infiltrating T lymphocytes or in neighboring epithelial cells in the disrupted epithelia remains to be determined. Testing an oncolytic effect of cutavirus on CTCL will require further evidence of its replicative potential in T cells in vivo and in vitro.

Conflict of interest

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.06.013.

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