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HIV-1 VIRAL DIVERSITY AND RESISTANCE IN CENTRAL NERVOUS SYSTEM BY DEEP-SEQUENCING

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BACKGROUND

- Although HIV-associated dementia (HAD) has decreased since the introduction of combined antiretroviral therapy (cART), milder forms of HIV-associated neurocognitive disorders (HAND) remain prevalent.
- Central nervous system (CNS) is one of several sites in which compartmentalized HIV-1 replication has been observed.
- The principal cause of HIV-1 persistence in the CNS is considered to be weak penetration of cART through the blood-barrier which may lead to selection of drug-resistant viruses.
- A previous study using Sanger sequencing showed independent evolution of drug resistance mutations to Nucleoside and Non-Nucleoside reverse Transcriptase Inhibitors (NRTIs/NNRTIs) and Protease Inhibitors (IP) in diverse regions of the CNS during antiretroviral therapy, suggesting that different drug penetration may occur among brain areas.
- Both Sanger and Single Molecule Real Time (SMRT) sequencing also reported uniquely divergent viral populations among frontal, occipital, parietal, temporal lobes and basal ganglia.
- SMRT sequencing also showed difference of co-receptor usage; brain envelope gene were predicted to use CCR5 co-receptor (R5-tropic), while most of non-brain envelope sequences were predicted to use CXCR4 co-receptor (X4-tropic).
- Most of studies assessed viral compartmentalization and diversity via cerebrospinal fluid or with Sanger sequencing and thus exhaustive information about tissue compartmentalization and diversity of HIV-1 is still limited.

OBJECTIVES

- In twelve post-mortem specimens derived from different brain areas of three HIV-1 patients diagnosed with probable or certain HIV-encephalopathy and using ultra-deep sequencing (UDS) technology, this study aimed:

(i) to describe thoroughly HIV-1 diversity in the CNS by sequencing reverse transcriptase (RT) gene and hypervariable V3 loop region of the gp120 envelope protein

(ii) to detect minority resistant variants

MATERIALS AND METHODS

- **Patients and tissues.**

Table 1: Participants' characteristics

Patient	Age (years)	Brain histologie	Year and cause of death	cART	Selected brain areas
1	48	Multinucleated giant cells (MGC) => Certain HIV-encephalopathy	2006 Pulmonary embolism	Multiple	Temporal lobe (TL) Caudate nucleus (CN) Cerebellum (Cer) Spinal cord (SC)
2	38	Numerous microglial nodules despite rare toxoplasma cysts => Possible HIV-encephalopathy	2010 Sepsis/ARDS	None	Temporal lobe (TL) Thalamus (Tha) Cerebellum (Cer) Medulla oblongata (MO)
3	29	Numerous microglial nodules despite rare toxoplasma cysts => Possible HIV-encephalopathy	2007 Pulmonary embolism	Unknown	Frontal lobe (FL) Caudate nucleus (CN) Cerebellum (Cer) Substantia nigra (SN)

ARDS: acute respiratory distress syndrome; cART: combination antiretroviral therapy

- **DNA quantification.** HIV proviral-DNA was quantified with the Generic HIV DNA Cell® kit (Biocentric).

- **RT and V3 gene amplification and UDS sequencing.** RT and V3 genes were amplified by nested PCR. Because of short reads generated by Illumina® technology, RT region was amplified as two separate parts, RT1 (450 bp) and RT2 (480 bp). Finally, sequencing was performed on Illumina® MiSeq (paired-end, 2x300 bp) in order to obtain at least 100 000 sequences.

- **Analysis of viral diversity.**

(i) Geneious Prime Software was used to trim reads by keeping those with Q-score>30 and of more than 200 bp length as well as to pair forward and reverse reads to form whole RT1, RT2 and V3 regions.
(ii) Sequences with 100% of agreement were grouped to form consensus sequences, then second round was performed with 99% of agreement allowed, then 98% and finally 97%.
(iii) Multiple alignments of all consensus sequences was performed using Mafft.
(iv) Phylogenetic analysis was performed using approximately-maximum-likehood method with FastTree 2.1 on both all consensus sequences (HIV_RT1_CS/HIV_V3_CS) and consensus sequences after cleaning viral consensus sequences found less than 100 times in each brain area (HIV_RT1_CS100/HIV_V3_CS100).
(v) HIV-1 tropism was determined with geno2pheno according to the recommendations from the European Consensus Group on clinical management of HIV-1 tropism testing (10% FPR).

- **Analysis of single nucleotide polymorphisms (SNPs).**

(i) Forward and reverse cleaned reads of RT1 and RT2 were mapped against annotated HXB2 to identify SNPs.
(ii) Position of synonymous and non synonymous SNPs, coverage and number of reads carrying each SNPs were determined.
(iii) HIV-1 genotypic drug resistance algorithms 2018 administered by ANRS, were used to identify resistance mutations.

RESULTS

Quantification of HIV proviral-DNA and viral diversity (Table 2 and Figure 1)

Table 2: HIV-reservoir quantification and viral diversity among the different brain areas

Patient	Brain area	HIV proviral-DNA (copies/ml)	HIV_RT1_CS No of CS	HIV_RT1_CS Consensus sequences shared	HIV_RT1_CS100 No of CS	HIV_RT1_CS100 Consensus sequences shared
1	Temporal lobe	< 40	1086	1 with CN	2	0
	Caudate nucleus	< 40	2590	15 with SC 1 with TL	141	2 with SC
	Cerebellum	< 40	Not amplified	-	Not amplified	-
	Spinal cord	< 40	3453	15 with CN	158	2 with CN
2	Temporal lobe	91	3259	3 with MO	146	0
	Thalamus	< 40	Not amplified	-	Not amplified	-
	Cerebellum	< 40	Not amplified	-	Not amplified	-
	Medulla oblongata	31	3189	3 with TL	72	0
3	Frontal lobe	544	2624	527 with CN and Cer. 55 only with CN 83 only with Cer.	30	7 with CD and Cer. 3 only with Cer.
	Caudate nucleus	130	2451	527 with FL and SN 234 only with Cer. 55 only with FL	60	7 with FL and Cer. 9 only with Cer.
	Cerebellum	23	3526	527 with FL and CN 234 only with CN 83 only with FL	73	7 with FL and CN 3 only with FL 9 only with CN
	Substantia nigra	92	1650	0	15	0

HIV_RT1_CS: all cleaned consensus sequences; HIV_RT1_CS100: all cleaned consensus sequences of RT1 fragment after filtering out sequences found less than 100 times.

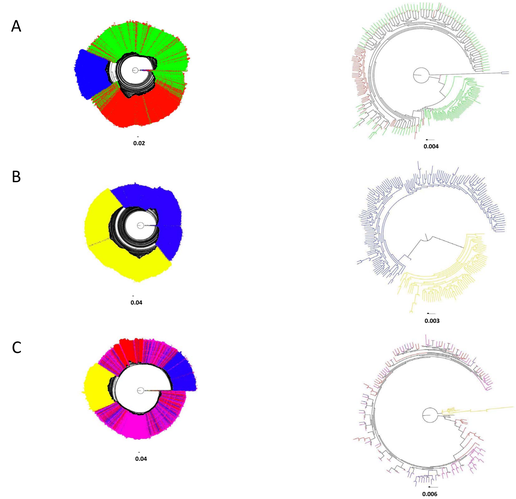

Figure 1: Approximately maximum-likelihood phylogenetic trees constructed with Fastree of RT1 consensus sequences issued from different brain area of P1 (A), P2 (B) and P3 (C). *Phylogenetic trees were inferred with all viral consensus sequences (HIV_RT1_CS, left) or viral consensus sequences filter out those found less than 100 times (HIV_RT1_CS100, right).*
Red: caudate nucleus; blue (P1 and P2: temporal lobe, P3: frontal lobe); green: spinal cord; yellow: brainstem (P2: medulla oblongata and P3: substantia nigra); pink: cerebellum.

- The majority of brain areas harbored a distinct HIV subpopulation wit high diversity.
- However, some variants isolated from CN were intermixed to various degree with sequences from SC, FL or Cer region.
- On the other hand, brainstem (SN and MO) area harbored a specific subpopulation in both patient 2 and 3.

HIV-1 tropism.

Only P1 temporal lobe and spinal cord were analyzed. High diversity was evidenced (Figure 2) as RT1 region and most of viral variants in both brain area were predicted to use CCR5 co-receptor (TL: 96%; SC: 94% of viruses).



Figure 2: Approximately maximum-likelihood phylogenetic trees constructed with Fastree of V3 consensus sequences issued from temporal lobe or spinal cord. *Phylogenetic trees were inferred with all viral consensus sequences (HIV_V3_CS, left) or viral consensus sequences filter out those found less than 100 times (HIV_V3_CS100, right).*
Blue: frontal lobe; green: spinal cord

SNPs and resistance mutations to NRTIs/NNRTIs.

- Synonymous and non-synonymous SNPs were presents for all samples in RT1. They were carried by either majority or minority variants depending on brain area and reflecting the viral diversity previously described.

- SNPs conferring resistance to ZDV, ABC, TDF/FTC (NRTIs) and ETR (NNRTIs) on condition that they were associated to other mutation of the RT gene were found (table 3).
- Prevalence of each mutation differed between different brain area of P1 and P3.
- In P2, no resistance mutations were identified either in temporal lobe or in medulla oblongata.

TABLE 3: Resistance mutations within RT1 fragment of patient 1, 2 and 3

PATIENT 1					
	Temporal lobe	Caudate nucleus		Spinal cord	
Resistance mutation		M41 L	V90 I	M41 L	V90 I
Nucleotide substitution (position)		A-C (586)	G-A (733)	A-C (586)	G-A (733)
Coverage (number of reads)	-	289 139	552 684	384 658	618 797
% Reads carrying mutation		98,4	96,7	98,4	97,6
number of reads carrying mutation		284 513	534 445	378 503	603 946
PATIENT 2					
	Temporal lobe			Medulla oblongata	
Resistance mutation	-			-	
PATIENT 3					
	Frontal lobe	Caudate nucleus		Cerebellum	Substantia nigra
Resistance mutation		V90 I	V106 I		M41 L V90 I
Nucleotide substitution (position)		G-A (733)	G-A (781)		A-C (586) G-A (733)
Coverage (number of reads)	-	328 900	328 913	-	62 637 100 327
% Reads carrying mutation		16,2	1		2,3 1,6
Number of reads carrying mutation		53 282	3 289		1 441 1 605

M41L: methionine substitution for leucine in position 41; V90I: valine substitution for isoleucine in 90; V106I: valine substitution for isoleucine in position 106

CONCLUSION

This study is the first to use Illumina® technology to study HIV diversity in the CNS reservoir from patients with HIV-encephalopathy. Our work show significant inter-regional and intra-regional diversity reflecting persistent viral replication. It also confirms HIV compartmentalization in different brain areas already shown by studies in classical and SMRT sequencing suggesting that there is not a single but several reservoirs within the CNS.