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Concordance of HIV Type 1 Tropism Phenotype to Predictions Using Web-Based Analysis of V3 Sequences: Composite Algorithms May Be Needed to Properly Assess Viral Tropism

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Abstract

Genotypic prediction of HIV-1 tropism has been considered a practical surrogate for phenotypic tests and recently an European Consensus has set up recommendations for its use in clinical practice. Twenty-five antiretroviral-experienced patients, all heavily treated cases with a median of 16 years of antiretroviral therapy, had viral tropism determined by the Trofile assay and predicted by HIV-1 sequencing of partial *env,* followed by interpretation using web-based tools. Trofile determined 17/24 (71%) as X4 tropic or dual/mixed viruses, with one nonreportable result. The use of European consensus recommendations for single sequences (geno2pheno false-positive rates 20% cutoff) would lead to 4/24 (16.7%) misclassifications, whereas a composite algorithm misclassified 1/24 (4%). The use of the geno2pheno clinical option using CD4 T cell counts at collection was useful in resolving some discrepancies. Applying the European recommendations followed by additional web-based tools for cases around the recommended cutoff would resolve most misclassifications.

Determination of viral tropism is a necessary step prior to the use of CCR5 antagonists and may provide clues in HIV pathogenesis. Genotypic assays are interesting alternatives to phenotypic assays, and although different interpretations of genetic data have been suggested, this issue is still unresolved. Recently a European consensus proposed the use of genotypic data. They suggested the use of the geno2pheno clonal option, with a false-positive rate (FPR) of 20%, to predict tropism on a single population genome and a 10% cutoff is recommended to predict tropism based on replicates.[1]

The evaluation of the concordance of phenotype determination of HIV-1 coreceptor usage to genotypic prediction has been an objective of different studies.[2–6] The sensibility and specificity of genotype prediction are influenced by different factors including viral subtype and the prevalence of CXCR4 using variants in the population assessed.[2,7,8] Among phenotypic assays, the Trofile is a reference. It is the only Clinical Laboratory Improvement Amendments (CLIA) certified assay, and its upgraded ESTA version has a high performance, with reported sensibility to X4 variants as low as 0.3% (http://www.trofileassay.com/what_is_trofile.html). Some aspects, however, limit its widespread use, including cost, sample transport logistics, a number of nonassayed samples, the inability to assess cell-associated genomes, and level of viremia necessary to run the assay (1000 copies/ml).

Although the actual correlation of the genotypic prediction with clinical response should be the main objective of these tests, that is, the assays should predict the clinical usefulness of CCR5 antagonists and not just show intraassay comparability; these data are limited and complex to analyze, as therapy success depends on many factors.[6] Therefore phenotypic assays are still important to validate genotypic predictions. In this study we compare the results of viral tropism as determined by the phenotypic Trofile ESTA assay to different genotypic tools.

Patients with virological failure on antiretroviral therapy, considering CCR5 antagonists as part of salvage therapy, were consecutively included. Two sets of patients were studied, paired samples, with two EDTA tubes obtained at the same blood drawn, at the clinical site and some additional unpaired cases, in which blood collection for the genotype assay was done before Trofile collection. Informed consent was obtained from all volunteers. V3 sequences were obtained with a nested PCR of the partial *env* genome as previously

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described from bulk virion RNA ($n=19$) or cell DNA ($n=8$, samples 6, 7, 18, 19, 20, 21, 22, and 23).[9] The V3 region was directly sequenced from PCR products in both directions. Fasta contigs were generated from two to four primers, with manual editing by Sequencher 4.6 software, considering nucleotide ambiguities (accession number JN 541241-65). The V3 sequence harboring nucleotide ambiguities was translated in all possible amino acids. Sequences with three or more amino acid mixtures due to nonsynonymous nucleotide ambiguities within the V3 region were not submitted to PSSM and net charge analyses. HIV-1 subtyping of partial *env* sequence was performed at NCBI and Rega websites.

The V3 sequences were interpreted according to different genotypic tropism predictions, including the 11/25 rule, which is based on the presence of arginine, histidine, or lysine at positions 11 or 25 of the V3 loop and two bioinformatics methods, PSSM (htpp://ubik.microbiol.washington.edu/computing/pssm), X4/R5 option, that analyze the composition and position of amino acids at the V3 sequence, generating a score concerning the likelihood of using the CXCR4 coreceptor and the Geno2pheno coreceptor (http://coreceptor.bioinf.mpi-sb.mpg.de/cgi-bin/coreceptor.pl), a statistical method based on the sequences FPRs, the likelihood of a sequence being mistakenly classified as a CXCR4.[10,11]

Sequences FPR were obtained using both clonal and clinical options, the latter using recommended nadir CD4 T cell (TCD4) counts and also TCD4 at time of collection, along with viral load; cell counted with flow cytometry (BD, USA) and viral RNA with and B-DNA (Siemens, USA).

Tests were run blind to the other results, and both were delivered to a reference physician who could use either information to subsidize clinical management. Reports from Trofile were reported as R5, X4, or dual/mixed tropism. Our report to clinicians designated the predicted tropism as R5, X4, or possible X4, using a composite rule. Trofile results were reported as R5-tropic, X4, and dual/mixed-tropic. In this study we dichotomized the cases as X4 (including dual/mixed and possible X4) or R5.

A total of 25 patients were included in this study, mostly (76%) males, with a median age of 39 years. All patients were in virological failure under antiretroviral therapy, with a median TCD4 of 218 cells/mm^3 and viral load of 10,000 copies/ml. Study cases consisted mostly of heavily treated patients, treated for a median of 16 years, having lived with HIV for a long time. The majority had a clade B envelope, with one case clade F (Sample 21). Samples were collected from December 2008 to October 2009 for Trofile tests, with 17 cases of paired and 8 cases of unpaired samples, with collection for genotyping from 2007 to 2009. Table 1 describes patients' demographic and clinical data.

Most HIV-1 infections were of X4-tropic viruses, 17/24 (71%), using the Trofile assay, whereas the IAL criteria showed 16/25 (64%), with one nonreportable case by Trofile. The FPR geno2pheno were obtained in three options—one clonal and two clinical evaluations: (1) using the last TCD4 available at the time of tropism test collection and (2) using nadir TCD4, as recommended. Most cases showed an FPR below 20%: 15/25 (60%), 14/24 (58%), and 13/17 (77%), respectively. It should be noted that the lack of information at collection or the nadir TCD4 did not allow the use of the clinical option for 1/25 (4%) and 8/25 (32%) cases, respectively. According to the "11/25 rule" 10/25 (40%) and at PSSM 9/22 (41%) were classified as X4-tropic viruses. The net charge ranged from 4 to 9. However, three samples (12%) with more than three nonsynonymous amino acid possibilities due to nucleotide ambiguities were not evaluated by PSSM: samples 8, 10, and 19. Table 2 describes individual data of the study cases, with the V3 alignments along with the Trofile result and interpretations of major algorithms. Table 3 compares the Trofile results assuming it to be the gold standard for defining disease (X4 or dual/mixed) or "no disease," involving only the R5 viral populations, to evaluate different determination algorithms. The overall concordance between distinct genotypic tools and ESTA ranged from 50% (Geno2pheno$_{10clinicalNadir}$) to 95.8% (IAL criterion), with a sensitivity and specificity for the detection of X4 variants using genotypic-based algorithms, as compared to Trofile, varying according to genotypic algorithms. The lower sensitivity was 37.5% Geno2pheno$_{10clinicalCollection}$. However, the specificity was adequate for many algorithms, including the 11/25 rule, Motivate, Geno2pheno$_{10clinicalcollection}$, PSSM, and IAL criteria.

The population studied included patients with advanced disease, exposed to multiple ARV regimens that were considering the use of CCR5 antagonists as part of the salvage regimen. Therefore, this should explain the high prevalence of X4 and X4/R5 dual-tropic viruses, when compared with others studies.[2,7] The study conclusions are limited by the small sample size and the use of a single sequence instead of replicates to predict tropism, but some points are relevant and deserve attention. The fact that the genotypic evaluation was

Table 1. Clinical and Demographic Data of Patients Included in the Study According to Trofile

	R5	*DM/X4*	*Total*
Age* (years)	45 (41–48)	18 (13–40)	39 (15–45)
Gender (% males)	86	76	76
TCD4* at collection (cells/mm^3)	226 (95–277)	184 (94–372)	218 (94–327)
Nadir TCD4* (cells/mm^3)	37 (17–79)	35 (27–97)	36 (23–108)
Viremia* at collection (log$_{10}$/ml)	3.43 (2.53–4.19)	4.47 (3.59–4.92)	4 (2.98–4.76)
Number of regimes*	8 (7–9)	6 (5–9)	8 (5–9)
Time on ARV* (years)	15 (13–16)	16 (4–17)	16 (13–17)
N (%)	7 (28)	17 (68)	25[a] (100)

[a]One case of Trofile not reportable, reported as R5 by the IAL rule.

Patient characteristics according to Trofile result, R5 and dual/mixed or X4 tropism, including age at the time of collection, percentage of males, TCD4 counts at collection, and the nadir value, documented throughout follow-up, plasma viremia (viral load) at collection, number of different ARV combinations (regimens) used by the patient and the total time on ARV therapy in years, expressed as medians and 25th–75th IQR percentiles*, and the total number of cases in each group. ARV, antiretroviral.

Table 2. Trofile and Genotypic Tropism Prediction of Study Cases

Samples	*Trofile*	*IAL criteria*	*G2F clonal FPR (%)*	*G2F clinical cFPR (%)*	*G2F clinical nFPR (%)*	*Rule 11/25*	*PSSM*	*Net charge*	*V3 sequence*
1	R5	R5	44.8	49.1	23.2	R5	R5	5	CTRPNNNTRKSIHMGWGRAFYATGDIIGDIRQAHC
2	D/M	R5	48.4	12.6	12.6	R5	R5	6	CVRPNNNTRKSIHMGWGRAFYATGEIIGNIRQAHC
3	R5	R5	22.3	26.3	8.2	R5	R5	(5-4)	CTRPNNNTRKGIHMG[MV]GRAFYATGEIIGEIRQA[HY]C
4	D/M	X4	4.8	48.4	19	X4	X4	5	CTRPNNNTRKSIPVGSGRILYATGKLIGDIRQAYC
5	Not reportable	R5	42.8	62.6	38.2	R5	R5	8	CTRPNNNTRRGIHMGPGKAFYATGNIIGNIRKAHC
6	D/M	X4	0.5	0.2	0.1	X4	X4	6	CTRPNNNTIRGIRIGPGRAVLATERIIGDIRRAHC
7	R5	R5	20.2	19.0	4.9	R5	R5	6	CTRPNNNTRKSVHIGPGSALYTTNIIGNIRRAHC
8	D/M	X4	3.2	11.9	6.8	R5	—	—	CTRP[x]N[x]TRKSIH[x]G[x]GRAF[x][x][x]G[x]VIG[x][x][x]QAHC
9	R5	R5	93.6	13.4	—	R5	R5	4	CTRLNNNTRRSIQMGWGRAFYATGDIIGDIRLAHC
10	D/M	X4	7.8	71	—	R5	—	—	CARPNNNTRK[SI]I[QH]MGW[RG]RAFYATGDI[IV]GDIRQAHC
11	D/M	X4	14.6	14.8	—	R5	R5	4	CERPSNNTRKGIHLGPGRAFFATEAIIGDIRQAHC
12	R5	R5	84.9	37.4	15.7	R5	R5	6	CTRPNNNTRKGIHMGWGRTLYATGAIIGDIRQAHC
13	D/M	X4	11.4	39.9	11.4	R5	R5	7	CTRPNNNTRKSIHMGAGRAFYTNGQIIGNIRQAHC
14	R5	R5	7.9	85.7	76.4	R5	R5	5	CTRPGNNTRKGIHIGPGRGAFYATDITGDIRQAHC
15	X4	X4	20.8	1.8	1.5	R5	X4	8	CTRPDYYAHKSINMLWGRRFHATGALKGNIKHL
16	R5	R5	57	51.4	8.8	R5	R5	5	CTRPSNNTRRSIHMAAGRALYTTDIIGDIRQAHC
17	D/M	X4	2.5	13	13	X4	X4	8	CTRLNNLTRRSIRIGPGGAWYAAGRIVGKIRPAHC
18	X4	X4	0.7	0.1	—	X4	X4	6	CTRPNNNTRKRVTMGPGRVWYTTGEIVGDIKRAHC
19	X4	X4	21.5	1.8	—	X4	—	—	CTRPNNNTRKS[x]HLGW[x]RT[x][x][x]T[x][x]IIGDI[x][x]A[x]C
20	X4	X4	15.6	15	—	R5	R5	4	CTRPNNNTRRSITIGPGRAFYGTDIIGDIRQAHC
21	X4	X4	1.8	17	—	X4	X4	9	CTRPNNNTRKSIHIGLGHAFR[TA]TEKIIGNIRKAHC
22	X4	X4	0	—	—	X4	X4	8	CTRPGNKTGKRIRIGHIGPGRTFYTTEKIRDIRQAHC
23	D/M	X4	18.3	23.2	23.2	X4	R5	(7-5)	CTRPNNNTRKGIHIGPGRSFYATG[KE]IIGDIRQAHC
24	D/M	X4	1.7	0.4	0	X4	X4	6	CSRPNNNTRKGISIGPGRAVYATEKIIGNIRQAHC
25	D/M	X4	0.1	0	0.4	X4	X4	7	CTRPHNNTRKRLYSSRWRTLYATE[IV]I[GV]GDIRQAHC

Data from each case including Trofile result, IAL composite interpretation, geno2pheno false-positive rates (FPR) of clonal and clinical option using TCD4 at collection TCD4 (cFPR) and nadir TCD4 (nFPR), 11/25 rule, PSSM, net charge, and the V3 sequence. Possible amino acids at positions with ambiguous nucleotides are shown as [MV]; cases 8 and 19 were not resolved [X] due to excess ambiguities.

Table 3. Concordance, Sensitivity, and Specificity of Genotyping Tools Taking as Reference the Trofile Assay

Genotypic algorithms	*Concordance (%)*	*Sensitivity (%)*	*Specificity (%)*
11/25 rule	70.8	59	100
$Geno2pheno_{Motivate}$	66.7	53	100
$Geno2pheno_{20clonal}$	79.2	76	85.7
$Geno2pheno_{20clínicoC}$	73.9	75	71.4
$Geno2pheno_{20clínicoN}$	68.8	90	33.3
$Geno2pheno_{10clonal}$	66.7	58.8	85.7
$Geno2pheno_{10clínicoC}$	69.6	37.5	100
$Geno2pheno_{10clínicoN}$	50.0	50	50
PSSM	76.2	64	100
IAL criterion	95.8	94	100

Agreement between Trofile results with different genotypic algorithms; geno2pheno were evaluated using different cutoffs. Values of false-positive rate, including 5.75, (motivate), 10%, and 20%, using both clonal and clinical option, the latter applying both recommended nadir TCD4 (N) and TCD4 at collection (C).

conducted in a public health laboratory setting is notable and documents the feasibility of the test in resource-limited settings.

The reliability of the genotypic methods may be influenced by polymorphisms found in Brazilian clade B sequences.[12] HIV-1 clade B GWGR bearing isolates, an uncommon motif in the V3 loop worldwide but with an important prevalence in Brazil, was observed in 28% of isolates, including one case with discordant genotype/Trofile result.

The use of replicates, as suggested by the European consensus, should further improve prediction. It is important to note that this discordant case (sample 2, Table 2) was re-evaluated as replicates without resolving the discrepancy.

The use of individual prediction algorithms showed, as in previous studies, a robust specificity but a suboptimal sensibility.[2,13] Using the geno2pheno clinical option with nadir TCD4 we obtained the best sensitivity score, 90%. The specificity, however, is low (Table 3). A recent study also suggests the usefulness of geno2pheno clinical option prediction.[4] In this study we further evaluated the clinical option using both the nadir and the TCD4 at the time of the collection. This has provided some additional information, as discussed below.

Overall, the analysis of the sequence set showed a high specificity with most web-based methods, higher than most reports in the literature.[2,14] Although this could be secondary to the small sample size evaluated here, with our discordance increasing as more samples are analyzed, many studies did not use the new ESTA Trofile assay. The earlier version of Trofile, and possible other phenotypic assays, may lack the high sensibility of ESTA to X4. These tests would therefore miss some X4 in paired, comparative studies. The lack of one or more X4 predictions by the "gold standard" would imply that some real X4 cases, identified by a genotypic algorithm with high specificity as PSSM and 11/25 rule, would be considered false-positive results, when they were actually performing better than the comparable phenotypic assays. Although plausible, this cannot be confirmed at this point but should be considered when interpreting these studies.

In spite of the fact that individual algorithms do not show a good concordance to the Trofile results, mostly due to low sensibility, the use of a "composite" rule, as applied by our service, improves prediction. Previous studies have also suggested that a combination of bioinformatics tools could improve the sensitivity and specificity.[15] The use of the European Consensus in this study would lead to 4/24 (16.7%) misclassifications (samples 2, 14, 15, and 19). In this sense, the use of $Geno2pheno_{20Clonal}$ could perform better if used in combination with other available genotypic prediction tools. In our analyses, a reliable prediction of X4 virus is obtained for samples with an FPR value below 5.75% (motivate cutoff). For R5, all but one case (sample 2) would be resolved with a cutoff of 22%; above 50% would yield a 100% concordance to Trofile. However, values within this range can benefit from other genotypic tools to improve the prediction, and the use of highly specific criteria, such as the "11/25 rule" and PSSM as well as the clinical option of geno2pheno, can be useful in this range. To illustrate, samples 3, 7, 15, and 19, all with a clonal FPR above 20%, would be considered R5 by European consensus. This would wrongly classified 2/4 (50%) (samples 15 and 19) (Table 2). The use of clinical geno2pheno (applying either nadir TCD4 or the last available TCD4 determination) may help resolve cases in this range. If clinical FPR is incorporated in the analysis, using collection TCD4, the two R5 have FPRs around 20% (26.3% and 19%), whereas the two X4 have an FPR below 5.75%. Additionally, these clinical FPRs are in concordance with PSSM and the results of the 11/25 rule. It is of note that the use of the clinical option with the nadir TCD4 would not resolve cases 3 and 7, as both show a low FPR at this option (8.2% and 4.9%).

The usefulness of these additional resources is also suggested by case 23, in which the clonal FPR, near the cutoff, can be further supported by incorporating the 11/25 rule. The most intriguing case is the discordant sample 2, with a high clonal FPR of 48.4%. This case has R5 in both the 11/25 rule and PSSM, but a low FPR (12.6%) at the clinical option that was not considered at the time of the test report.

Another situation is cases with an FPR below 20% but above 5.75%. Most (5/6) cases would be correctly classified as X4 by the European consensus recommendation, but by considering clinical option results, over 75%, along with R5 by PSSM and 11/25 rules, would resolve the single discordance. Although highly predicted for this study, these rules and cutoff suggestions should be improved and validated by larger studies. However, our data do not support the assumption that a single parameter, albeit practical, may be adequate for the prediction of genotypic tropism for Brazilian patients with advanced disease.

On the other hand, the study also suggests that a composite evaluation of existing tools may prove robust in predicting HIV coreceptor tropism.

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