BRIEF COMMUNICATION

Tumour necrosis factor haplotypes associated with sensory neuropathy in Asian and Caucasian human immunodeficiency virus patients

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Key words
TNF haplotype; immunogenetics; neuropathy; HIV; NRTI; inflammation

Abstract

In human immunodeficiency virus (HIV) patients, neuropathy is a common adverse side effect to some antiretroviral treatments, particularly stavudine. As stavudine is cheap, it is widely used in Asia and Africa. We showed that increasing age and height moderately predict the development of neuropathy. This was improved by the inclusion of tumour necrosis factor (TNF)-1031 (rs1799964). To investigate this association, Malay (n = 64), Chinese (n = 74) and Caucasian patients (n = 37) exposed to stavudine were screened for neuropathy. DNA samples were genotyped for polymorphisms in the central major histocompatibility complex (MHC) near TNF, and haplotypes were derived. The haplotype group FVa6,7,8 (incorporating TNF-1031) was found to be associated with neuropathy in Chinese patients in bivariate analyses (P = 0.03), and in Malays and Chinese in a multivariate analysis correcting for age and height (P = 0.02, P = 0.03, respectively). This trend was also confirmed in Caucasians.

Since the introduction of antiretroviral therapy, sensory neuropathy (SN) has become the most common neurological complication suffered by patients with human immunodeficiency virus (HIV) (1). SN may be promoted by exposure to nucleoside analogue reverse transcriptase inhibitors (NRTIs), notably stavudine (2). During 2009, stavudine was administered as a first line therapy to 98% of HIV patients in Johannesburg (3). NRTI-associated sensory neuropathy (NRTI-SN) is clinically and histologically similar to distal sensory polyneuropathy (DSP), a subset of SN directly caused by HIV. NRTI-SN is distinguished from other forms of SN by a temporal relationship between symptom onset and exposure to NRTIs (4). Here, we sought to investigate the factors predicting the development of neuropathy after exposure to stavudine.

SN is typically characterised by pain in the feet described as ‘aching’, ‘painful numbness’ or ‘burning’ (5). Patients may also have reduced or absent ankle reflexes, hyperalgesia and allodynia. The pathogenesis of the DSP involves disordered inflammation, with activated macrophages and cytokine production including tumour necrosis factor-α (TNF-α), observed around the peripheral nerves in affected patients (6, 7). There is in vivo and in vitro evidence that aberrant host inflammatory responses underpin painful neuropathies in other settings (8). While there is no direct evidence for inflammation playing a role in NRTI-SN, an association with genes affecting inflammation in our preliminary work (9, 10) provides circumstantial evidence.

Major histocompatibility complex (MHC) haplotypes defined by human leukocyte antigen (HLA) and TNF alleles associate with susceptibility to numerous inflammatory diseases, including rheumatoid arthritis, type 1 diabetes and sepsis (11–13). Carriage of the minor allele of a
TNF promoter region single nucleotide polymorphism (SNP; TNF-308*2) occurred at a higher frequency in Caucasian and African-American HIV-infected adults with dementia, than in HIV-infected adults without dementia or a healthy control group (14, 15). We associated SNP within this region, including TNF-1031*2, with NRTI-SN risk in Australian-Caucasian patients exposed to potentially neurotoxic NRTI (10). This SNP also associates with SN in stavudine-exposed Indonesian HIV patients (9). However, the SNP responsible for disease phenotype has not been identified, because strong linkage disequilibrium (LD) creates conserved ‘TNF block’ haplotypes.

To identify SNPs responsible for various disease phenotypes, ‘TNF block’ haplotypes were reconstructed using 38 SNP spanning 45 kb around the TNF (16) in healthy Caucasian, Australian-Aboriginal and Asian donors. Using the phase algorithm (17, 18), we defined a total of 31 haplotypes (denoted FV1–31). A different combination of 12–18 FV haplotypes was present in each ethnicity, of which 11 contained the SN-associated SNP TNF-1031*2 (rs1799964). To narrow down the list of SNPs affecting neuropathy, we selected six SNPs located in the BATI intron and promoter regions (rs9281526, rs2523506 and rs2844509), NFKIBL promoter regions (rs2230365) and TNF promoter regions (rs1799964 and rs1800629), which sorted the 11 haplotypes containing TNF-1031*2 into five groups, denoted as FVa (Table 1, Panel A). These were typed in stavudine-exposed Caucasian, Malay and Chinese HIV patients of known SN status to define the TNF alleles associated with SN. As it is a cross-sectional design, the phenotype does not distinguish DSP from neuropathy induced by stavudine.

HIV patients who had been exposed to stavudine were screened for neuropathy using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screening Tool (ACTG BPNS) (19). All patients who had received stavudine therapy at least for 2 months were included at the Pokdisus AIDS Clinic at the Cipto Mangunkusumo Hospital, Jakarta, Indonesia (n = 92), and at the Infectious Diseases Clinic at the University of Malaya Medical Centre, Kuala Lumpur, Malaysia (n = 46). Neuropathy was defined by the presence of both symptoms and signs on the ACTG BPNS. Screened patients were separated according to self-proclaimed ancestry: Chinese (n = 74) and Malay (n = 64). Laboratory, clinical and demographic parameters were collected from medical records, and a DNA sample was collected from saliva. Genotyping was performed by an operator who was unaware of the neuropathy status of the samples.

TNF genotyping was also available in a cohort of Caucasian HIV patients (n = 37) previously defined as having NRTI-SN (onset within 6 months of first exposure to stavudine, didanosine or zalcitabine) or NRTI-SN-free (no symptoms or signs of SN despite at least 6 months of exposure to these NRTIs) (10). TNF haplotypes were also constructed in this cohort, and data were compared with findings from Asian populations.

Control DNA samples were collected from 181 Caucasian (from the Busselton Survey, Western Australia) and 99 Chinese (largely Southern Han, recruited from Kuala Lumpur, Malaysia, and Perth, Western Australia) donors. Malay populations were represented by 136 donors living in Kelantan, Malaysia. The study was approved by all relevant Human Research and Ethics Committees and all participants gave informed consent.

DNA sample was extracted from saliva or blood leukocytes using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) and stored at −80°C. Six SNPs were genotyped using TaqMan FAM or VIC-labelled probes (C26778958,10, C2273595,10, C1613476,10, C7514871,10 and C7514879,10 for rs2523506, rs2844509, rs2230365, rs1799964 and rs1800629, respectively) (Applied Biosystems, Foster City, CA, USA) and Universal PCR Master Mix (Applied Biosystems, Foster City, CA). rs9281526 was genotyped using a custom TaqMan assay (20). DNA samples were diluted to 10 ng/μl and amplified on an ABI Prism 7900HT (Applied Biosystems) in 384-well microtitre plates. Thermocycling involved one step of 10 min at 95°C, followed by 40 cycles of denaturation at 92°C for 15 s with annealing and extension at 60°C for 1 min. Fluorescence was read on an ABI 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA) using proprietary allelic discrimination software. Repeats and non-template controls were included in each run. The median (range) genotyping success rate was 99% (98%–100%) for each SNP in all cohorts.

Allele and genotype frequencies in each ethnic group conformed with the Hardy–Weinberg equilibrium (HWE) when assessed using GENEPOP v3.3 (21). The phase algorithm (17, 18) was used to statistically infer mini-haplotypes that were annotated FVa to match our earlier publication (16). Demographic details and mini-haplotypes were compared with groups using chi-squared tests (dichotomous variables), Wilcoxon rank-sum tests [non-normally distributed continuous variables, described using median and interquartile range (IQR)] or unpaired t-tests [normally distributed continuous variables, described using mean ± standard deviation (SD)]. Logistic regression modelling was used to identify the factors independently associated with SN risk. All factors associated with SN risk on bivariate analysis (P < 0.1) in any cohort were included, and a step-wise removal procedure was used to obtain the final model shown. Statistical analyses were performed using Stata 9.2 (Stata-Corp, College Station, TX).

HIV patients were divided according to ethnicity (Malay, Chinese and Caucasians) and grouped by neuropathy status as neuropathy positive (HIV+ SN+) and neuropathy negative (HIV+ SN−). Comparisons were made with healthy controls (Table 1, Panel A). When the SNPs were considered individually, TNF-1031 (rs1799964) in Chinese patients was the only SNP where carriage of the minor allele differed between patients with and without SN [P = 0.01, odds ratio (OR) = 4.4]. Allele frequencies were similar in Chinese...
Table 1  Population frequencies of haplotypes carrying TNF-1031*2 (rs1799964) in HIV+ SN patients and controls\textsuperscript{ab}

<table>
<thead>
<tr>
<th>Panel A</th>
<th>Malay</th>
<th>Chinese</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype</td>
<td>TNF</td>
<td>FVa1,3,4,12</td>
<td>FVa6,7,8</td>
</tr>
<tr>
<td>n = 21</td>
<td>n = 43</td>
<td>n = 136</td>
<td>n = 89</td>
</tr>
<tr>
<td>TNF-1031*2</td>
<td>47.7%</td>
<td>36.6%</td>
<td>48.1%</td>
</tr>
<tr>
<td>FVa1,3,4,12</td>
<td>0%</td>
<td>0%</td>
<td>5.2%</td>
</tr>
<tr>
<td>FVa6,7,8</td>
<td>0%</td>
<td>0%</td>
<td>32.6% \textsuperscript{cd}</td>
</tr>
<tr>
<td>FVa9,10</td>
<td>14.3%</td>
<td>7.0%</td>
<td>11.1%</td>
</tr>
<tr>
<td>FVa24</td>
<td>0%</td>
<td>2.3%</td>
<td>0%</td>
</tr>
<tr>
<td>Cumulative frequency</td>
<td>47.6%</td>
<td>23.3%</td>
<td>48.7%</td>
</tr>
<tr>
<td>2n</td>
<td>42</td>
<td>86</td>
<td>272</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel B</th>
<th>Malay</th>
<th>Chinese</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>P</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.02</td>
<td>1.18</td>
<td>1.02–1.36</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.01</td>
<td>1.11</td>
<td>1.03–1.21</td>
</tr>
<tr>
<td>FVa6,7,8</td>
<td>0.03</td>
<td>5.02</td>
<td>1.19–21.0</td>
</tr>
<tr>
<td>Overall model P value</td>
<td>0.002</td>
<td>0.01</td>
<td>NA</td>
</tr>
</tbody>
</table>

CI, confidence interval; HIV, human immunodeficiency virus; OR, odds ratio; SN, sensory neuropathy; TNF, tumour necrosis factor.

\textsuperscript{a}Bivariate analysis suggested that FVa6,7,8 may affect SN status (Panel A). Multivariate analysis comparing genotyping and age, height and FVa6,7,8 between stavudine-exposed HIV patients with and without sensory neuropathy yielded a significant model in Chinese and Malays (P = 0.007, P = 0.02, respectively) (Panel B). Haplotypes not found in any populations were omitted. NI denotes SNPs not included in analyses. The major allele at each SNP is denoted '1' and the rare allele at each SNP is denoted '2'. NS denotes P ≥ 0.2 in bivariate analyses and therefore excluded from the multivariate model. NA denotes incomplete data.

\textsuperscript{b}The cumulative FVa haplotype and rs1799964 minor allele frequencies were measured by carriage. In some cases, a few donors were homozygous for the minor allele of rs1799964 but carried different FVa haplotypes, accounting for the lower values recorded for rs1799964. A few carriers of rs1799964 had no resolvable haplotype, accounting for some instances where the allele frequencies were higher than haplotype frequencies.

\textsuperscript{c}P < 0.05 using Fisher exact test comparing healthy Malay controls with healthy Chinese controls.

\textsuperscript{d}P < 0.05 using Fisher exact test comparing healthy Malay controls with healthy Caucasian controls.

\textsuperscript{e}P < 0.05 using Fisher exact test comparing HIV+ SN+ with HIV+ SN− cohorts.
The six SNPs were typed in all patients and controls, and (rs2230365) and NFKIBL haplotypes defined by Valente et al. (16). Four 6-SNP-haplotypes were named FVa1,3,4,12, FVa6,7,8, and FVa9,10 was more common in Malay than healthy patients (P = 0.04, OR = 2.22) (Table 1, Panel A). Carriage of FVa9,10 was more common in Caucasian HIV+ SN+ patients compared with controls (P = 0.05, OR = 2.22) (Table 1, Panel A), but controls and HIV+ SN− patients were otherwise similar. However, several ethnic differences were apparent. Carriage of FVa9,10 was less common in Malay HIV patients compared with Caucasian HIV patients (P = 0.006, OR = 4.6); correspondingly, carriage of FVa6,7,8 was more common in Malay than healthy Chinese (P = 0.0003, OR = 3.47) and Caucasian controls (P = 0.001, OR = 3.46), and more common in Malay HIV patients than in Chinese HIV patients (P = 0.02, OR = 2.89) (Table 1, Panel A).

In bivariate analyses (Table 1, Panel A), FVa6,7,8 conferred a sevenfold increased risk of NRTI-SN in Chinese patients (P = 0.03, OR = 7.1). Malay and Caucasian patients also displayed similar trends. In general, carriage of FVa6,7,8 was similar in HIV+ SN+ patients and controls, and lower in HIV+ SN− patients. This difference was significant amongst Malays (P = 0.01, OR = 2.9). FVa9,10 appeared to be associated with a reduced risk of SN in Caucasians, but this was not evident in a multivariate analysis. Among the clinical risk factors considered, including age, height, gender, CD4 nadir and current counts, it was found that age was associated with SN across all ethnicities (0.001 < P < 0.07), and height was associated with SN in Malay patients (P = 0.02).

Logistic regression modelling showed that FVa6,7,8 was independently associated with NRTI-SN risk in both Malay and Chinese cohorts after correcting for patient age and height (Table 1, Panel B). It conferred an increased SN risk (P = 0.03, OR = 7.7) in ethnic Chinese HIV patients and Malays (P = 0.02, OR = 5.0). A similar trend was observed in the smaller Caucasian cohort when the same model was applied (P = 0.16, OR = 8.7) (Table 1, Panel B).

Here, we investigate the association between TNF-1031*2 carriage and stavudine neuropathy (9, 10), and define TNF block haplotypes carrying this allele associated with NRTI-SN in multiple ethnic groups. A role of TNF in neuropathy is biologically plausible as activated macrophages and elevated levels of TNF-α have been found in the peripheral nerves of HIV patients with SN (7). Furthermore, it has been shown that intraneural injection of TNF-α produced major axonal damage of sciatic nerve in mice (22). As CD4+ T-cell counts did not affect neuropathy here (data not shown) or in other studies that we have published (10), it is unlikely that the observed effects of TNF haplotype on neuropathy arise because the haplotypes affect the severity of HIV disease. A further two SNPs in the IL-1 (rs17561) and IL-12 (rs3212227) genes were genotyped based on previous studies (10); however, these SNPs were not significantly associated with SN incidence in any of the cohorts.

Although TNF-1031*2 carriage has previously been associated with NRTI-SN, LD within the TNF block means that this may not be a causal association. Of the 11 TNF haplotypes studied, FVa6,7,8 carriage was associated with an increased risk of neuropathy in Chinese and Malay stavudine-exposed patients after correcting for age and height. FVa6,7,8 was rare in Caucasians, but a similar trend was evident with this and the more common FVa9,10 haplotype. In Asians, the haploptic association (P = 0.03, OR = 7.06) was clearer than the association with TNF-1031 (P = 0.04, OR = 3.16). This suggests that TNF-1031 is not solely responsible for the effect on SN. TNF-1031*2 may be in LD with the causal SNP, or several SNPs in or near the TNF haplotype FVa6,7,8 (possibly including TNF-1031*2) may promote SN in HIV patients exposed to stavudine. Of the complete 38 SNP haplotypes, FVa6,7,8 carriers carries SNPs within the BAT1 promoter (rs2523506, rs2251824), NFKIBL promoter (rs3219186), TNF promoter (rs2857713, rs1799964, rs4248158) and the LST exon 5 (rs1052248) regions. Currently, there are no functional studies on these SNPs.

There are a number of limitations to this study. First, sample sizes were relatively modest so there may be type 2 errors. Second is the possible misclassification of the NRTI-SN status, particularly among Malay and Chinese cohorts. No data were available regarding the timing of symptom onset relative to stavudine use in these groups, and patients with isolated symptoms or signs were classified as ‘neuropathy free’. However, misclassification of neuropathy status was independent of the risk factors considered and so can only dilute our findings. Significant findings among Malay and Chinese cohorts are strengthened by confirmation in the smaller but more rigorously defined cohort of Caucasians.

This work shows that TNF haplotypes independently associate with NRTI-SN in Chinese, Malay and Caucasian patients. SNPs common to FVa6,7,8 and FVa9,10 are credible candidates for further association studies.

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References