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GNB3 C825T Polymorphism and Response to Anti-retroviral Combination Therapy in HIV-1-infected Patients – A Pilot Study

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Abstract
Methods: Analyses were based on a cohort of 55 HIV infected patients whose treatment was changed due to therapy failure. Viral load, $CD4^+$ and $CD8^+$ cell counts were determined before therapy and after 8 weeks and 16-32 weeks, respectively. *GNB3* genotyping was performed using Pyrosequencing. Chemotaxis of purified $CD4^+$ cells was quantified in a Boyden chamber using stromal cell-derived factor 1α (SDF-1α) as a stimulus.
Results: Age gender, route of infection, treatment, and baseline values for viral load and $CD4^+$ and $CD8^+$ cell counts were not associated with *GNB3* genotypes (10 TT, 24 TC, 21CC). After 8 weeks of treatment viral load was lowest in TT genotypes (log copies/ml: TT: 1.88 ± 0.32; TC: 2.49 ± 0.24; CC: 3.06 ± 0.22; p = 0.039) and this trend tended to persist until final visit. Unexpectedly, TT genotypes had lowest $CD4^+$ cell counts at final visit (TT: 165.3 ± 34.0; TC: 414.4 ± 72.1; CC: 441.5 ±70.9; p = 0.047). SDF-1α -stimulated chemotaxis was reduced in $CD4^+$ cells from HIV-1-infected patients compared to healthy controls. Still cells from TT genotypes displayed strongest chemotaxis.
Conclusions: This pilot study suggest that the *GNB3* C825T polymorphism is associated with short-term success of HAART treatment in HIV-1-infected patients.

Key words: Signal transduction; genetics; AIDS; HAART

Introduction

The susceptibility for infection with HIV-1, the natural course of the disease as well as the response to highly-active anti-retroviral therapy (HAART) are significantly influenced by genetic host factors (for review see ref. [1]). As HIV-1 entry into cells critically depends on chemokine receptors [2], most investigations have so far focused on functional single nucleotide polymorphisms (SNPs) in genes encoding chemokine receptors and their natural ligands, e.g. CCR5 [3], CCR2 [4], CX3CR1 [5], stromal-cell derived factor 1α (SDF-1α) [6], and RANTES [7], to name only a few. Intracellular signal transduction through chemokine receptors is mediated through ubiquitously expressed heterotrimeric G proteins which consist of α,- β, and γ subunits [8]. Whether entry of HIV-1 into lymphocytes and its intracellular replication requires solely co-receptor usage or also involves G protein-mediated signal transduction is controversially discussed in the literature [9-13].

The T allele of a common C825T polymorphism in the gene *GNB3* which encodes the β3 subunit of heterotrimeric G proteins is associated with alternative splicing of the gene and the generation of functionally active splice variants [14, 15]. Our own studies suggest that G protein signaling is enhanced in cells from 825T allele carriers: Both neutrophils [16] and $CD4^+$ lymphocytes [17] from 825T allele carriers display an enhanced chemotaxis on stimulation by cytokines and this effect can be mimicked by transfection of the splice variant Gβ3-s into cells [16]. Moreover, the proliferation of polymorphnuclear cells in response to stimulation by common recall antigens is significantly enhanced [17]. Finally, cells from individuals with a 825T allele show an increased *in vitro* activity against a hepatitis B antigen following in vivo booster vaccination of probands against the hepatitis B virus [18]. Collectively, these investigations suggest that certain aspects of the strength of the human cellular immune response at least in part depend on the efficacy of G protein activation associated with C825T genotype status.

In the present study, therefore, we assessed whether *GNB3* genotypes might be associated with the efficacy of HAART in HIV-1 infected individuals. For that purpose we enrolled 55 consecutive HIV-1 patients in a observational study which aimed at investigating potential genotype effects on typical outcome parameters of HAART, i.e. changes in $CD4^+$ and $CD8^+$ cell count and in HIV-1 viral load. In addition, we determined potentially genotype- dependent chemotaxis of $CD4^+$ cells from HIV-infected individuals. Our findings suggest an impact of *GNB3* genotypes on response to HAART in HIV-1- infected patients.

Materials and Methods

Patients

Subjects were enrolled in our study on presentation in our HIV outpatient clinic. We recorded the time of first diagnosis of HIV-1 infection while the actual time of infection or seroconversion were unknown. The sole parameter for inclusion into the study was a change therapy due to side effects or treatment failure. Thus, this study design may reflect the typical clinical situation in which therapy is adapted according to clinical needs and in which therapy success is monitored through measurement of viral load and monitoring of $CD4^+$ and $CD8^+$ lymphocyte count. These parameters were determined at the start of therapy, 8 weeks thereafter, and after a time period of 16 – 24 weeks, this time point being referred to as "last visit". Data for viral load and cell counts were only included if they fell into the chosen time periods. The study started with 55 patients, at last visit 43 patients were still in the study. Sole reason for loss of follow-up was non-attendance. Characteristics of the patients are given in Table 1. Viral genotypes as a reason for potential drug resistance were not determined in the present study.

The study is part of and founded by the German competence network on HIV/AIDS. The protocol was approved by the ethical committee of the Ruhr-University Bochum and all patients gave written informed consent before inclusion into the study.

Laboratory Parameters

$CD4^+$ and $CD8^+$ lymphocyte counts were measured by standard techniques. Plasma HIV-1 RNA levels (viral loads) were determined using the Roche Amplicor Monitor Assay (Roche Diagnostics, Penzberg, Germany) using either the standard method or the ultrasensitive adaptation. *GNB3* genotypes were determined by Pyrosequencing™ as described [19].

Isolation of $CD4^+$ T Lymphocytes and Chemotaxis Assay

Venous blood was obtained from healthy blood donors and from HIV-1 seropositive patients ($CD4^+$ T cell count: 864 ± 128 cells x 10^6 cells/ml, all > 300 x 10^6 cells/ml, without significant difference between genotypes). Peripheral blood mononuclear cells (PBMCs) were immediately isolated by centrifugation through Ficoll-Paque™ (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient. The PBMC suspension was washed with RPMI 1640, $CD4^+$ T-lymphocytes were prepared by negative selection with Cellect™ cell enrichment immunocolumns (Cytovax, Canada) according to the manufacturer's instructions. Chemotaxis of $CD4^+$ T-lymphocytes was determined in a 48-well microchemotaxis chamber as described with minor modifications: chemoattractants were diluted in RPMI 1640 containing 0.1 % fatty-acid free BSA, and the cell suspension was adjusted to a density of 2.0 x 10^6 cells/ml in RPMI 1640 containing 1.0 % fatty-acid free BSA. Fifty µl of the cell suspension were applied to the upper wells of the microchemotaxis chamber separated from the lower wells by a polyvinylpyrolidone-free polycarbonate filter membrane with a pore size of 5 µm coated with fibronectin (6.7 µg/ml overnight). Migration was allowed for 2 h in a humidified atmosphere of 5% CO_2 at 37 °C. Migrated cells were quantified on the lower side of the filter after staining with Diff-Quick® by counting three random areas of each well under a microscope with a magnification of 400-fold (high power field, HPF) - the mean ± SD for each well was calculated. To determine random migration (chemokinesis), chemoattractants were also added to the cells in the upper wells of the microchemotaxis chamber in some experiments.

Statistical Analysis

Values of viral load measurements were log-transformed to give log10 copies/ml after adding the value "1" to all data. As the lower detection limit was 40 copies/ml, patients with a test result of <40 copies/ml were assigned half of the detection limit, i.e. 20 copies/ml. If not otherwise indicated all values given are means ± SEM.

Results from chemotaxis assays were compared using Student's t-test. Dichotomous variables were compared using chi^2 statistics. Comparison of variables over all genotypes was performed using the Kruskal-Wallis test or the Mann Whitney test if only two genotype groups were compared. Changes before and after treatment were assessed using the Wilcoxon signed rank test. The level of significance α was set at 0.05.

Results

Patient Characteristics

Characteristics of the patients and their treatment are summarized in Table 1. The majority of patient was of male gender (89 %) and most of them had acquired HIV-1 infection through homo- or heterosexual contacts. Other routes of infection included e.g. intravenous drug abuse. Disease stage according to the Center of Disease Control (CDC) 1993 criteria and treatment regimens were not significantly different by genotype. In general patients received a nucleoside reverse transcriptase inhibitor as backbone in combination with other nucleoside reverse transcriptase inhibitors, with protease inhibitors or with non-nucleoside reverse transcriptase inhibitors.

Viral Load

Baseline viral load averaged 4.20 ± 0.15 log copies/ml and was not significantly different between genotypes (Table 2, Fig.1A). After 8 weeks of treatment, viral load decreased significantly to 2.59 ± 0.15 log copies/ml ($p < 0.0001$). However, viral load was significantly different by genotype ($p = 0.015$; Table 2; Fig. 1B). Most remarkably, viral load was lowest in TT genotypes, followed by TC and CC genotypes. Accordingly, decreases in viral load averaged 2.38 ± 0.43, 2.11 ± 0.22, and 1.23 ± 0.28 log copies/ml for TT, TC, and CC genotypes ($p = 0.04$), respectively. Thus,

Table 1. Patient characteristics by *GNB3* genotype.

	All	TT	TC	CC	p-value
Gender (m/f)	49/6	9/1	22/2	18/3	0.81
Age at first diagnosis (yrs)	41.3 ± 1.3	40.7 ± 2.2	40.3 ± 2.1	42.7 ± 2.1	0.45
Age at start of therapy (yrs)	43.9 ± 1.3	41.7 ± 1.9	43.1 ± 2.1	45.8 ±2.1	0.46
Route of infection					
Heterosexual (n)	10	1	5	4	
Homosexual (n)	36	5	15	16	
Other (n)	9	4	4	1	0.17
CDC classification (A/B/C)	14/ 13 /28	1 / 4 / 5	7 / 7 / 10	6 / 2 / 13	0.26
Therapy					
3 NRTI	12	2	5	5	
NRTI / PI	24	5	9	10	
NRTI / NNRTI	19	3	10	6	0.89

m, male; f, female; yrs, years; CDC, center of disease control; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; all values are means ± SEM. P-values are derived from chi2 statistics or Kruskal-Wallis tests.

Table 2. Viral load and $CD4^+$ and $CD8^+$ T-cell count by *GNB3* genotype.

	All	TT	TC	CC	p-value
Viral load Start	4.20 ± 0.15 (53)	4.26 ± 0.24 (9)	4.54 ± 0.19 (23)	4.00 ± 0.23 (21)	0.31
Viral load Week 8	2.59 ± 0.15 (53)	1.88 ± 0.32 (9)	2.49 ± 0.24 (23)	3.06 ± 0.22 (19)	0.039
Viral load Last Visit	2.18 ± 0.25 (42)	1.49 ± 0.28 (7)	2.73 ± 0.42 (19)	1.75 ± 0.36 (16)	0.14
$CD4^+$ Start	294.1 ± 31.3 (55)	206.7 ± 74.6 (10)	332.8 ± 54.3 (24)	291.6 ± 39.7 (21)	0.29
$CD4^+$ Week 8	372.4 ± 36.5 (55)	232.8 ± 73.2 (10)	401.3 ± 56.2 (24)	405.7 ± 59.4 (21)	0.14
$CD4^+$ Last Visit	378.1 ± 44.0 (43)	165.3 ± 34.0 (8)	414.4 ± 72.1 (19)	441.5 ± 70.9 (16)	0.047
$CD8^+$ Start	1010.0 ± 71.9 (55)	812.8 ± 187.3 (10)	1037.0 ± 111.4 (24)	1073.0 ± 107.8 (21)	0.36
$CD8^+$ Week 8	1057.0 ± 71.9 (55)	1097.0 ± 205.6 (10)	979.8 ±95.3 (24)	1126.0 ± 122.2 (21)	0.77
$CD8^+$ Last Visit	1071.0 ± 87.0 (43)	1171 ± 218.1 (8)	1008.0 ± 93.1 (19)	1095.0 ± 162.8 (16)	0.84

Viral load is given in log10 copies/ml, $CD4^+$ and $CD8^+$ cell counts are x 10^6 cells/ml. Number in parentheses refer to number of patients from whom data were available. P-values are derived from Kruskal-Wallis tests. Data are means ± SEM.

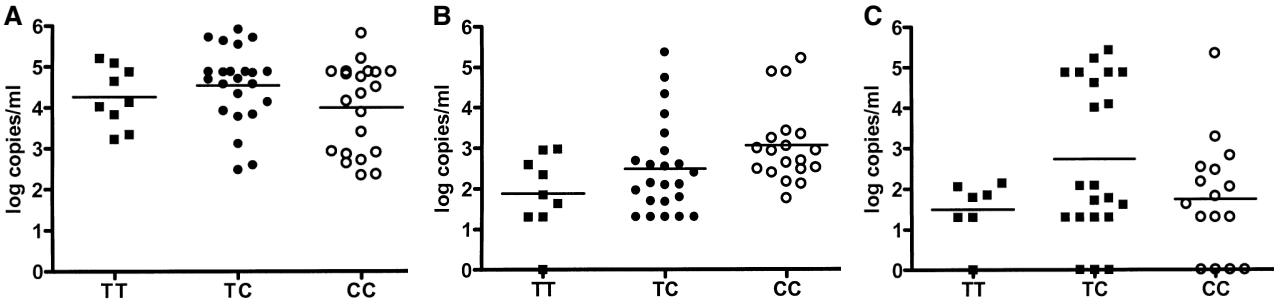

Fig. 1. GNB3 genotype and effect of HAART on plasma viremia. HIV-1 copies are shown at the start of therapy (panel A), after eight weeks (panel B) and at last visit (panel C) after log10 transformation of original values. Horizontal lines indicate mean values. ■, TT genotype; ●, TC genotype; O, CC genotype; p-values from Kruskal-Wallis- tests. Each point represents one single patient.

825T allele carriers showed the strongest decreases in viral load despite similar pretreatment values. Final measurements of viral load yielded no significant differences by genotype (Table 2, Fig.1C). However, there was still a trend for TT genotypes to present with the lowest values of viral load. Interestingly, data points of Fig.1C suggests a dichotomous distribution of log copies selectively in patients with TC genotype with 9 out of 21 patients displaying values of 4 log copies/ml or more, and with 5 patients presenting with an in-

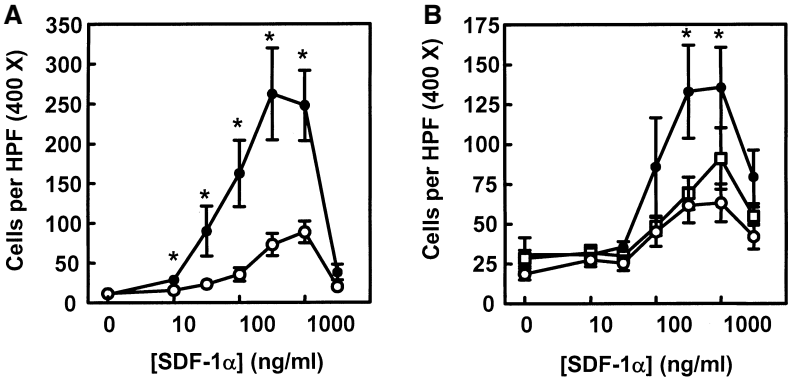

Fig. 2. Chemokine-stimulated chemotaxis of $CD4^+$ T-cells. Chemotaxis was stimulated with the indicated concentrations of stromal cell-derived factor 1a (SDF-1α). A, cells from healthy, HIV-1-negative blood donors with *GNB3* CC (O; n = 5) or TC/TT (●, n = 5; 4 TC, 1 TT) genotype. B, cells from HIV-infected individuals with *GNB3* CC (O, n = 6), TC (□, n = 7), or TT (●; n = 5) genotype. *, $p < 0.05$ versus CC genotype. Data are given as migrated cells per high power field (HPF; x 400).

creased viral load compared to week 8. This effect was not observed in TT or CC genotypes.

$CD4^+$ and $CD8^+$ Cell Count

Baseline $CD4^+$ cell counts were not significantly different by *GNB3* genotype although there was a trend for lowest values in TT genotypes (Table 2). After eight weeks mean $CD4^+$ cell counts were still not significantly different by *GNB3* genotype. However, we observed different capacities to increase $CD4^+$ cell counts dependent on genotype by comparison of matched values before and after treatment. In TT genotype the increase in $CD4^+$ cell count, after week 8 was statistically not significant ($p = 0.08$) and averaged 26.1 (95 % CI: -65.2 to + 117.4) x 10^6 cells/ml. A significant increase by 68.5 (95 % CI: 12.9 to 124.2; ; $p = 0.026$) x 10^6 cells/ml was observed for TC genotype, and the highest increase by 114.1 x 10^6 cells/ml (95 % CI: 55.2 to 173.0; $p = 0.0001$) was seen in CC genotype. Thus, the strongest recovery of $CD4^+$ cell count after eight weeks was associated with the CC genotype. A similar result was obtained at the last visit at which $CD4^+$ cell counts were significantly different by genotype (Table 2). Again, compared to baseline no significant difference? 34.8 x 10^6 cells/ml (95 % CI: -81.3 to 150.8; $p = 0.25$) was seen in TT genotypes. In TC genotype the increase averaged 73.4 x 10^6 cells/ml (95 % CI: 8.2 to 138.5; $p= 0.07$), and in CC genotype the increase averaged 153.5 x 10^6 cells/ml (95 % CI: 69.9 to 237.1; $p = 0.0021$). Thus, although the strongest decrease in viral load under HAART was associated with the TT genotype this effect was not accompanied by a corresponding recovery of the $CD4^+$ cell count.
$CD8^+$ cell counts were not significantly different by *GNB3* genotypes throughout the study (Table 2). However, there was a slight albeit statistically not significant trend suggesting an increase of $CD8^+$ cell count over time associated with the TT genotype (data not shown).

Chemotaxis of $CD4^+$ T-cells

SDF-1a - stimulated chemotaxis of $CD4^+$ T cells from healthy individuals was significantly enhanced in cells from homo- or heterozygous 825T allele carriers (Fig. 2A). In analogy to earlier findings on interleukin-8 - or fMLP- stimulated neutrophils and SDF-1α-stimulated lymphocytes [20], the presence of one 825T allele apparently suffices to fully establish enhanced chemotaxis. SDF-1a - evoked chemotaxis was generally reduced in $CD4^+$ T cells from treated HIV-1 seroprevalent individuals (Fig. 2B). Notably, we observed comparable chemotaxis of lymphocytes from individuals with CC and TC genotype, whereas chemotaxis in cells from individuals with TT genotype was still significantly enhanced.

Discussion

The aim of the present study was to determine a potential association of *GNB3* genotypes with treatment outcome in HIV-1-infected patients under HAART. This study was performed recruiting patients consecutively who presented with different stages of HIV-1 disease and in whom medication was changed due to side effects or treatment failure. Thus, it may be regarded a disadvantage of the study design that the studied cohort did not consist of treatment-naïve patients and the number of pretreatment cycles and drugs used previously are not known for all patients. A second potential drawback of the study is the fact that patients were not uniformly treated with a standard combination of drugs and therapy was adjusted according to clinical needs. On the other hand, the treatment situation used here for our genetic association study may closely mirror clinical practice in which patients are continuously treated and in which medication is adapted according to the individual needs.

From our association study two major observations can be reported: i. Decrease of plasma viremia after 8 weeks of treatment with HAART depends on *GNB3* genotype and is strongest in individuals with TT genotype despite practically identical initial viral load in all genotype groups. Changes in viral load during this period appears compatible with an allele-dose effect. Whether this effect persists over longer observation periods could not be investigated. After the end of the observation period (Fig. 1C), there was still a trend for lowest viremia in TT genotypes which, however, missed statistical significance probably due to drop-out of some patients. It is presently unclear how the high numbers of treatment failures in the TC group can be explained. Nevertheless, more effective clearance of HIV-1 associated with TT genotype is compatible with earlier observations suggesting stronger $CD4^+$ cell activation in 825T allele carriers [17] and the novel results presented here showing increased

chemotaxis of $CD4^+$ cells from HIV-1-infected subjects with *GNB3* TT genotype (Fig. 2B). We have no evidence to suggest that these findings are confounded by duration of infection with HIV-1, different pretreatment histories or genotype-associated differences in actual medication.

The second interesting finding of the present report is the fact that individuals with TT genotype may fail to significantly increase $CD4^+$ cell counts despite showing the most pronounced fall in viremia. It is commonly known that recovery of $CD4^+$ cell count under HAART depends on pretreatment values, and this increase is commonly higher in patients with higher initial values, i.e. less advanced disease. While TT genotypes entered this study with somewhat reduced initial $CD4^+$ cell counts compared to individuals with TC and CC genotype (Table 2), this difference was not statistically significant. On direct examination of pre- and post-treatment $CD4^+$ T cell counts it was even more surprising to observe statistically significant increases in patients with TC or CC genotype, while patients with TT genotype showed no significant increases. This observation, if replicated by others, could be clinically relevant, the underlying mechanisms, however, remains to be investigated.

We can speculate, however, that an increased proliferation and/or cytolytic activity of $CD8^+$ cells in TT genotypes may contribute to an increased clearance of HIV-1 infected cells through apoptosis of $CD4^+$ T cells, thereby keeping $CD4^+$ cell count low. How these observations relate to increased SDF-1α-stimulated chemotaxis of $CD4^+$ cells from HIV-1 infected patients with TT genotype is another unresolved matter. Chemotaxis in general was reduced in cells from HIV-1 infected patients compared to those from healthy controls suggesting that yet unidentified components of intracellular signal transduction are impaired by HIV infection. Intuitively one would expect that increased chemotaxis and other aspects of increased immune cell activation frequently seen in 825T carriers should provide an advantage also for HIV-1-infected 825T allele carriers. This is actually observed with regard to viral clearance but not with regard to $CD4^+$ cell recovery. Further investigations are required to resolve this question. Finally, the observations reported here raise the question whether *GNB3* genotypes are associated with an accelerated progression to AIDS and/or reduced survival of HIV-1 infected patients. It might be speculated that TT genotypes with their tendency of decreased $CD4^+$ cell count are at higher risk for AIDS than those with TC and CC genotypes. A study to resolve this problem is currently underway in our group.

While this appears the first report on *GNB3* genotypes associated with response to HAART, other genetic polymorphisms were previously associated with treatment success and results are still highly controversial. A common CCR5Δ32 polymorphism results in the formation of a truncated CCR5 receptor and may be associated with immunity against HIV-1 infection in homozygous individuals and retarded disease progression in heterozygous individuals [21-23]. While some authors reported an increased virological response in CCR5Δ32 carriers (24), others could not confirm these findings [25, 26]. Failure of viral suppression may be associated with a combination of different polymorphisms in the genes CCR5 and CCR2 [27]. Amino acid variations at codons 249 and 280 of the CX3CR1 receptor may be associated with immunological failure under HAART [28]. The human multi-drug-resistance gene (MDR-1) encodes the ATP-dependent P-glycoprotein efflux membrane transporter which is broadly expressed including $CD4^+$ T cells. A single C3435T polymorphism in exon 26 has been described [29], the T-allele being associated with decreased expression of MDR-1 transcripts in lymphocytes [30]. Moreover, overexpression of MDR-1 protein is associated with a reduced uptake of protease inhibitors, e.g. saquinavir, ritonavir, nelfinavir, and indinavir [31]. Fellay et al. reported an association between MDR-1 genotypes, plasma concentrations of nelfinavir and efavirenz and the kinetics of $CD4^+$ T cell recovery in HIV-1-infected patients, while the decrease in viral load was not associated with MDR-1 genotypes [30]. Others found no association between MDR-1 genotypes and time to virological success or virological or immunological failure, respectively [32, 33]. Taken together, multiple genetic polymorphisms may be associated with therapy success in HIV-1-infected patients and future investigations will have to define genotype combinations or haplotypes in order to optimally guide HAART.

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