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Dosage and Duration Effects of Korean Red Ginseng Intake on Frequency of Gross Deletions in the *nef* Gene

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In the present study, we investigated whether a gross deletion in the *nef* gene (gΔ*nef*) is induced by Korean red ginseng (KRG) intake. Ten patients were treated with KRG powder for 3 years in the absence of antiretroviral drug therapy. On average, 3,555$\pm$1,042 g KRG was administered per person over 36.1$\pm$2.4 months. There was a mild decrease in CD4 T cell count (75$\pm$110/μL) over the 36.1$\pm$2.4 months (p=0.059). We obtained 355 *nef* amplicons using 71 peripheral blood mononuclear cell samples over a 3-year period. All ten patients exhibited gΔ*nef* (range, 3.2 to 45.9%). At baseline, 3 of 78 amplicons (3.8%) exhibited gΔ*nef*, whereas 18.8% (52/277) revealed gΔ*nef* during KRG-intake (p<0.001). The proportion of gΔ*nef* was significantly correlated with monthly dose of KRG (r=0.89, p<0.001). The median time for first detection of gΔ*nef* was 13 months. In conclusion, our data show that gΔ*nef* is inducible by KRG intake and its proportion is dependent on the duration of KRG intake and dose of KRG.

Keywords: AIDS, HIV-1, *nef*, Korean red ginseng, Gross deletion

INTRODUCTION

Gross deletions or genetic defects in the human immunodeficiency virus (HIV)-1 gene in HIV-1 infected patients are very rare [1-3]. Thus, it is known that genetic defects may not be a common characteristics among long-term nonprogressors (LTNPs)/long-term survivors (LTS) or elite controllers. However, gross deletions in the *nef* gene (gΔ*nef*) have been reported in many LTNPs [4,5]. However, limitations in most of these studies, such as small patient numbers or absence of appropriate control groups, have hindered interpretation of results [6]. Highly active antiretroviral therapy (HAART) has reduced mortality and morbidity related to human immunodeficiency virus diseases [7]. However, treatment with all antiretroviral agents selects for drug-resistant mutations in the HIV-1 gene that ultimately result in therapy failure. To my knowledge, there is no report that describes any treatments that attenuate HIV-1 (or other) viruses.

Panax ginseng C. A. Meyer has been used as a drug for more than 2,000 years [8]. Recently, many reports put a high value on ginseng as immune modulator and adjuvant [9,10]. In 1991, we began treating HIV-1 infected patients with Korean red ginseng (KRG) for 6 months and observed that KRG-intake had various beneficial effects, including increases in CD4 and CD8 T cell counts [11-13]. We subsequently reported that KRG slows depletion of CD4 T cells in HIV-1-infected patients over 10 years [14-17]. We also showed that there was a high frequency of gΔ*nef* in LTS [18] and hemophiliacs [19] that had been treated with KRG; in this latter group, there was a dose-dependent relationship between KRG intake and the frequency of gΔ*nef*. In ad-

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dition, the frequency of a gross deletion in the 5'-LTR/gag in these KRG-treated patients was 2-fold higher than that of the g*Δnef* [20]. With KRG treatment alone, several patients among less than 100 HIV-1 infected patients diagnosed prior to 1991 remained healthy for 20 years after diagnosis [20,21], and we recently showed that combined treatment with HAART plus KRG was more beneficial than HAART only [22].

However, despite demonstrating a strong association between the occurrence of g*Δnef* and KRG intake, our previous study was unable to define the time at which the first gross deletion occurred during KRG intake due to the absence of baseline data and samples collected prior to 3 years after initiating KRG intake [18,19]. In the present study, we investigated the time to first occurrence of a g*Δnef* in ten patients for whom baseline and/or earlier samples were available. Our data show that g*Δnef* is inducible in all patients within 30 months of KRG intake and its proportion depends on the duration and dosage of KRG intake.

MATERIALS AND METHODS

Study population

Among our cohort, ten patients infected with subtype B of HIV-1 and treated with KRG were selected. Inclusion criteria were consistent intake of KRG for up to 3 years and availability of at least five samples for 3 years in the absence of HAART. We excluded hemophiliacs because these patients were infected with HIV-1 through a different mode of transmission [23]. Patients infected with non-subtype B were also excluded. All patients were diagnosed between 1989 and 2004 (Table 1). Except 2 patients (95-87 and 96-51), they had not taken KRG prior to this study. Eight patients were male and two were female. Informed written consent was obtained from all subjects before the initiation of the study.

Therapy with Korean red ginseng

KRG treatment in HIV-1-infected patients was begun on an outpatient basis at the Korean National Institute of Health in late 1991 [11,12]. The daily dose of KRG was 5.4 g for men and 2.7 g for women [22]. On average, 3,555 ± 1,042 g KRG was administered over the course of 36.1 ± 2.4 months.

CD4 T cell counts and plasma HIV-1 RNA copy numbers

Blood was drawn from each patient at 3- to 6-month intervals, and peripheral blood mononuclear cells (PBMCs) in each sample were incubated with phycoerythrin- and fluorescein isothiocyanate-conjugated antibodies (combined in the Simultest reagent; Becton Dickinson, San Jose, CA, USA) against CD4 and CD8 antigens, respectively. Levels of CD4 and CD8 T cells were measured by flow cytometry using FACScan (Becton Dickinson). Plasma concentrations of HIV-1 RNA were measured using the Amplicor HIV-1 Monitor kit (Roche Diagnostics, Branchburg, NJ, USA) [16].

Amplification of the *nef* gene

Proviral DNA was extracted from uncultured PBMCs. *nef* amplicons were amplified from each sample by double-nested (rarely, triple-nested) PCR as described elsewhere [18,19].

Table 1. Baseline characteristics of 10 HIV-1-infected patients and outcome

Patient code[1)]	Sex/age at diagnosis (yr)	Subtype of HIV-1	HLA class I	CD4 T cell (/uL)	Plasma RNA copy	Stat time of this study	Outcome at present
89-14	M/29	KSB	A2,24 B7,48 C7,8	222	ND	Jan 1992	Death
91-22	M/8	KSB	A1,24 B39,52 C7,12	316	9,400	Sep 2002	On KRG
92-48	M/47	KSB	A24, 31 B7, 54 C1, 7	287	ND	Oct 1992	Suicide
95-87	M/32	KSB	A2, 24 B48, 67 C7, 8	512	44,000	Jun 2005	On KRG
96-51	M/28	KSB	A2,- B51,61 Cw10,14	514	29,200	Jun 2000	On HAART/KRG
97-116	F/23	KSB	A2,- B59,67 C1,7	330	22,100	Jul 2001	On KRG
01-99	F/22	B	A2,- B46,51 C1,10	228	16,400	Apr 2001	On KRG
01-179	M/50	KSB	A31,33 B44,61 Cw10	407	134,508	Jul 2004	On KRG
03-493	M/45	KSB	A2,- B27,46 C1,5	716	7,671	Jan 2005	On KRG
04-397	M38	KSB	A24,- B35,- Cw9,-	450	530,000	Nov 2004	On HAART

Two digits prior to hyphen means year of HIV-1 diagnosis.
HIV, human immunodefi ciency virus; KSB, Korean subclade B of HIV-1; ND, not determined, however, p24 was positive in serum; KRG, Korean red ginseng; HAART, highly active antiretroviral therapy.
[1)]Patient code means number in national registry.

HLA typing

DNA was isolated from PBMC and HLA-A, -B, and -C typing was performed using the amplification-refractory mutation system-PCR method. Each tube contained a primer mix consisting of the allele- or group-specific primer pairs, as well as a plus control primer matching the nonallelic sequences. There were 32 sets specific for HLA-A, 27 sets for HLA-B, and 23 sets for HLA-C. Details are described elsewhere [16].

Statistical analysis

Data are expressed as means±standard deviations. Statistical significance was estimated by Student's two-tailed *t*-test, the chi-square test, Fisher's exact test, or correlation analysis, using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $p<0.05$.

Nucleotide sequences

GenBank accession numbers are HM369809-920, HM369933-040, AY121476, AY221717, DQ121935-948, AY121479, AY221649, AY363353-354, HM369962-970, HM747120, HM747122-123, HM747126-128, HM747130, HM747133, and HM747135-136.

RESULTS

Viral load and CD4 T cells

In the eight patients in whom plasma viral RNA copy number was determined (P24 antigen was measured in the remaining two patients) [24], there was a significant correlation between plasma viral RNA level and the decrease in CD4 T cells ($r=0.87$, $p<0.01$). In four of eight patients, viral RNA copy number was measured at both baseline and endpoints. In three patients, the changes (1,470 to 9,400 copies/mL, 44,000 to 30,730 copies/mL, and 22,100 to 34,839 copies/mL) were not significant. However, in one patient with a high baseline level of viral RNA (530,000 copies/mL), viral copy number increased to 4,755,746 copies/mL over the course of 33 months in association with a continuous decrease in CD4 T cell count from 450/μL to 190/μL. This steep decrease in CD4 T cell might be associated with homozygotes at 3 alleles of HLA class I (A24,-B35,- Cw9, -) and the worst prognosis allele B35 [25,26] (Table 2).

Relationship between Korean red ginseng intake and change in CD4 T cells

On average, patients consumed 3,555±1,042 g KRG (range, 1,512 to 5,520 g) over a time span of 36.1±2.4 months. The average monthly dose was 101±25 g (range, 63 to 154 g). Although there was a trend toward a decrease in CD4 T cell count (from 398±154/μL to 323±146/μL) over the duration of the trial, this corresponds to annual decrease of 24/μL ($p=0.059$).

Higher frequency of *nef* gene after KRG intake compared to baseline

Including 10 samples at baseline or prior to KRG intake, we collected 71 PBMC samples over 36.1±2.4 months from patients on KRG intake for PCR analysis of the g*Δnef*. Four reactions for each sample were at-

Table 2. Proportion and onset time of gross deletion in the g*Δnef* during KRG-intake

Patient	Monthly KRG (g)	No. of PBMC sample	No. of PBMC with g*Δnef*	No. of PCR amplicons	No. of g*Δnef* [1)]	Proportion of g*Δnef* (%)	Time on the first detection of g*Δnef* from KRG intake (mo)
89-14[2)]	109	8	5	35	12	34.3	0
91-22	79	5	1	18	2	11.1	4
92-48[2)]	154	8	6	37	17	45.9	0
95-87	90	6	2	29	2	6.9	12
96-51	120	4	3	18	5	27.8	18
97-116	63	7	1	31	1	3.2	17
01-99[2)]	107	5	3	23	4	17.4	0
01-179	88	6	3	30	3	10.0	14
03-493	108	7	2	35	4	11.4	29
04-397	90	5	2	21	2	9.5	14
Total	101±25	61	28	277	52	17.8±14	13

g*Δnef*, *nef* gene; KRG, Korean red ginseng; HIV, human immunodeficiency virus; PMBC, peripheral blood mononuclear cell.
[1)]Gross deletion in the g*Δnef* .
[2)] Three patients revealed one g*Δnef* at baseline, respectively.

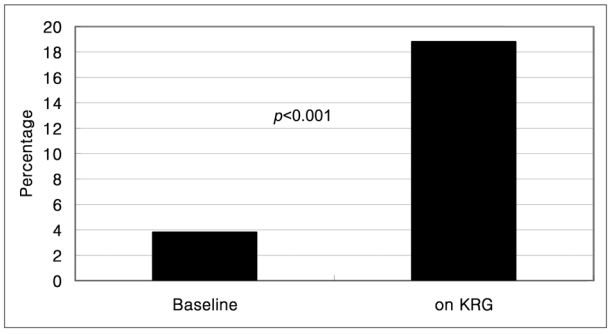

Fig. 1. Comparison of the proportion of PCR amplicons with gross deletions in the *nef* gene (gΔ*nef*). At baseline, the proportion of gΔ*nef* was 3.8% (3/78), whereas during Korean red ginseng (KRG) intake it increased significantly to 18.8% (52/277) ($p < 0.001$).

tempted. Of the 78 amplicons obtained at baseline, 3 amplicons (3.8%) from three patients were gΔ*nef* together with wild type. In detail, the three patients (89-14, 92-48, and 01-99) revealed one gΔ*nef* at baseline, respectively. The proportions were 12.5% (1/8), 9.1% (1/11), and 7.7% (1/13), respectively. During KRG intake, we obtained 277 amplicons from 61 PBMC samples. Of these, 52 amplicons (18.8%) from 28 PBMC samples (45.9%) were gΔ*nef* ($p < 0.01$) (Fig. 1).

Median time for the detection of first *nef* gene

All ten patients exhibited gΔ*nef* during KRG intake, with a median time for first detection of gΔ*nef* of 13 months (range, 0 to 29 months) (Table 2). The locations and sizes of gross deletion are described in Table 3.

Correlation between *nef* gene proportion, and dose and duration of KRG intake

The proportion of gross deletions as a percent of amplicons was variable among patients (3.2% to 45.9%). The patient (92-48) who revealed the highest proportion took the highest dosage of KRG. He was followed up to 53 months after the end of this study. Overall 50 amplicons were obtained during KRG intake and 25 were grossly deleted (50%), whereas there was one gΔ*nef* among 11 amplicons before KRG intake (1/11) ($p < 0.05$). There was a significant relationship between the monthly amount of KRG intake and the proportion of gΔ*nef* ($r = 0.89$, $p < 0.001$) (Fig. 2). To determine whether the proportion of gΔ*nef* was related to the duration of KRG intake, we divided all 355 PCR amplicons into three 12-month groups (baseline, 1 to 12 months, 13 to 24 months, and 25 to 36 months) according to the duration of KRG intake.

Importantly, we found that the proportion of gΔ*nef* increased with increasing duration of KRG intake ($p < 0.01$); gΔ*nef* proportion was 3.8% (3/78) at baseline, 9.6% (7/73) at 1 to 12 months, 20.4% (19/93) at 13 to 24 months, and 23.4% (26/111) at 25 to 36 months (Fig. 3).

No premature stop codon in the *nef* gene

We determined sequences of 140 wild type *nef* out of 295 amplicons obtained during KRG intake. There was no premature stop codon or G-to-A hypermutations. However, in patient 01-99, all sequences revealed deletion of 9 base pairs of 9th-11th amino acids from the initiation codon.

DISCUSSION

Previous report showed that KRG slows depletion of CD4 T cells in HIV type 1-infected patients. In addition, our studies over the last 10 years have indicated a strong association between KRG intake and gross deletion in the gΔ*nef* [18,19,21]. However, these previous reports have suffered from important limitations, including lack of baseline and earlier samples within 3 years after initiating KRG intake. Thus, although we were able to establish that the occurrence of gΔ*nef* depends on the amount of KRG intake [19], we could not determine the relationship between the duration of KRG intake and the detection of gΔ*nef*.

In the present study, we focused on the first 3 years of KRG intake. Considering a sampling interval of about 6 months, the median time for the detection of gΔ*nef* might be less than the 13 months estimated here. In any case, gΔ*nef* occurs considerably earlier than the median time 67-month of our previous study [18].

Among the three patients who revealed gΔ*nef* at baseline, patient 01-99 was infected with HIV-1 through her husband, who was diagnosed with HIV-1 infection in 1987 and had taken KRG since 1994, suggesting that the wife's gΔ*nef* might have resulted from previous virus exposure to KRG. The husband, who has remained healthy in the absence of HAART for 22 years, did not reveal gΔ*nef* at baseline [20], but did have a high proportion of gross deletion in 5'-LTR/gag [20]. Our data show that a high frequency of gΔ*nef* is directly associated with KRG intake rather than host factors or introduction of a genetically defective HIV-1 strain in Korea.

Regarding prognosis, except one patient 04-397 with the worst HLA allele, all patients show relative good prognosis. In detail, 91-22 who had been infected with HIV via transfusion in December 1989 remains healthy for 20 years in the absence of HAART. After this study

Table 3. Characteristics of gross deletions in the *nef* gene (gΔ*nef*)

Patient code	Day of samplin	No. of bands by PCR[1)]	No. of PCR reactions with gΔ*nef*[2)]	Size of Δ (bp)[3)]	Beginning nt. relative to HIV-1 NL4-3	D outside gΔ*nef* (bp)	GenBank accession No.
89-14	Jul 91	2	1	451	8,859		
	Dec 92	2	1	545	8,826		HM747120
	Jun 93	2	1	408	9,021		HM369815
		2	1	321	8,922		HM369818
	Dec 93	2	1	453	8,868		HM369824
	May 94	2	4	544	8,698	89env	
	Dec 94	2	4	544	8,698	89env	
91-22	Dec 02	1	1	744	8,798		AY221678
		1	1	511	8,882		AY221679
92-48	Oct 92	2	1	438	8,765	20env	
	Sep 93	2	2	504	8748	29env	HM747122
		2	1	297	8994		HM369874
	Dec 93	2	1	549	8698	89env	HM747123
	Jun 94	2	4	256	9060		HM369882
		2	2	352	8822		HM369883
	Dec 94	2	2	549	8698	89env	HM747126
	May 95	2	2	549	8698	89env	HM747127
		2	2	437	8819		HM369886
	Oct 95	2	1	452	8862		HM369888
95-87	May 07	2	1	373	8,871		HM369905
	Feb 08	2	1	324	8,913		HM369911
96-51	Apr 02	1	1	104	8,707	79env[4)]	DQ121943
	Dec 02	1	1	ND	ND		
	Oct 03	2	1	630	8,749	38env	DQ121946
		2	1	642	8,716	71env	DQ121948
97-116	Dec 02	2	1	331	8,764	13env	HM369950
01-99	Apr 01	2	1	242	8,916		HM369964
	Sep 02	1	1	211	8,895		AY21650
		1	1	312	9,030		AY221651
	Mar 03	2	1	252	9,055		HM369970
	Sep 03	2	1	636	8,751	25env	HM747130
01-179	Mar 05	1	1	497	8,873		DQ122080
	Mar 06	2	1	311	8,910		
	Sep 06	2	1	281	8,795		
03-493	Jun 07	2	1	379	9,040		HM370007
	Jun 08	2	3	544	8,698	89env	HM747133
04-397	Jan 06	2	1	624	8,783	3env	HM747135
	Sep 07	2	1	24	8,757	19env	HM747136

[1)]1 and 2 denote single short band only and wild type with gΔ*nef*, respectively.
[2)]Number of PCR products (of four PCR amplicons per sample) with gΔ*nef*.
[3)]Δ (bp) denotes deletion (base pair).
[4)]89env denotes 89-bp deletion in the *env* gene.

period, the patient revealed another gΔ*nef* (1/20) in June 2008 and gross deletion/premature stop codons in the *vif* gene (1/39) as well as in the 5'LTR/gag (7/26). Patient 97-116 are also healthy without decrease in

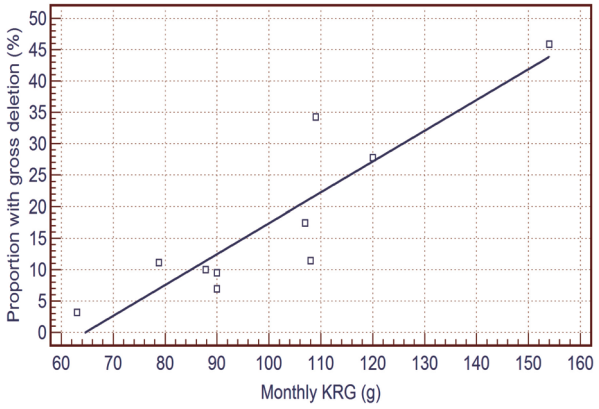

Fig. 2. Correlation between the monthly amount of Korean red ginseng (KRG) intake and the proportion of gross deletion in the *nef* gene during KRG-intake ($r = 0.89$, $p < 0.001$).

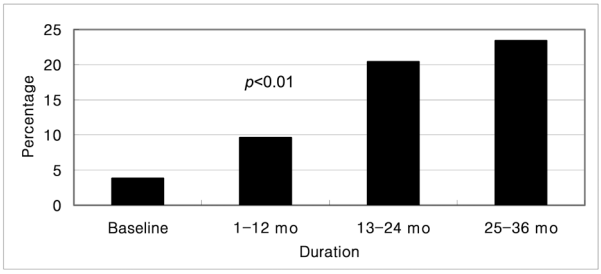

Fig. 3. Relationship between the duration of Korean red ginseng intake and the proportion of *nef* gene (gΔ*nef*). The proportion of gΔ*nef* was 3.8% at baseline, 9.6% at 1 to 12 months, 20.4% at 13 to 24 months, and 23.4% at 25 to 36 months ($p < 0.01$).

CD4 T cell for 13 years. Patients 96-51 and 03-493 had maintained CD4 T cell count more than 500/μL for 13 and 6 years, respectively. Patient 01-99 has maintained CD4 T cell count for 9 years. However, despite KRG and zidovudine therapy, patient 89-14 died of acquired immunodeficiency syndrome in May 1996 before introduction of HAART in Korea. Patient 92-48 committed suicide in 1998 on HAART. Patients 96-51 and 04-397 began to take HAART from September 2009 and September 2007, respectively.

Despite the association between KRG intake and gΔ*nef* we do not know the mechanism for the *nef* deletions. In the same patients, the proportion with gross deletion in 5'LTR/gag was 23.1% during KRG intake (data not shown). In addition, G-to-A hypermutations in the *vif* and *pol* genes are significantly high during KRG intake (data not shown). In other words, the gΔ*nef* is not the only gene affected by KRG intake. It seems that whole HIV-1 genes are nonspecifically affected by KRG intake [13,20,21]. Therefore, we can speculate that both direct antiviral and indirect immunological mechanisms are involved together with decrease in chronic immune activation in CTL function [12,16,17]. With respect to the antiretroviral effects of ginseng, several reports also support our data. It is known that several ginsenosides including polyacetyleneginsenoside-Ro from *Panax ginseng* and xylanase from *Panax notoginseng* inhibited the replication of HIV-1 [27-29]. In addition, it is known that ginsenosides Rb1, Rb2, Rb3, and Rc have anti-HIV replication in vitro (patent, CN1745756A).

Regarding immunological mechanism, it seems that gΔ*nef* might indirectly result from the enhancement of innate immunity than adaptive immunity [30-32], immune modulation toward Th1-cytokines [33,34], anti-inflammatory response through TLR4 induced NF-*k*B [35] as well as the attenuation of hyper-immune activation state [12,16]. In particular, it appears that the LTR region including gΔ*nef* is more fragile to KRG intake than are other regions of HIV-1 because HIV-1 gene expression is modulated by *cis*-acting regulatory elements in the LTRs [36]. In addition, approximately 200 substances, such as ginsenoside, polysaccharides, polyacetylenes, peptides, and amino acids, have been isolated from Korean ginseng [37]. Therefore, it might not be easy to elucidate the mechanism. Through this study, we confirmed that gΔ*nef* is inducible by KRG intake although future study is required whether the gΔ*nef* is caused by ginsenosides or polysaccharide.

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