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Frequent Genetic Defects in the HIV-1 5'LTR/*gag* Gene in Hemophiliacs Treated with Korean Red Ginseng: Decreased Detection of Genetic Defects by Highly Active Antiretroviral Therapy

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We investigated whether Korean red ginseng (KRG) and highly active antiretroviral therapy (HAART) affect the frequency of gross deletion in 5'LTR/*gag* in 20 hemophiliacs. This study is a prospective study in 20 hemophiliacs who were infected with Korean subclade B of HIV-1 from two cash-paid plasma donors in 1990. Over a 13-year period, we obtained 436 amplicons of 5'LTR/*gag* genes by nested polymerase chain reaction using 147 peripheral blood mononuclear cells. Of the 436 amplicons, 92 (21.1%) showed gross deletion in 5'LTR/*gag*. Despite of a 2.3-fold higher monthly dose of KRG intake, the frequency of gross deletion in 5'LTR/*gag* (16.4%) was significantly decreased during HAART compared with 28.1% prior to HAART ($p<0.01$). Gross deletion in 5'LTR/*gag* was 10% more detected on KRG-therapy than prior to KRG-therapy ($p<0.05$). In addition, we also obtained 28 amplicons containing premature stop codon or isoleucine at initiation codon of 254 amplicons sequenced on KRG intake (7.5%) or HAART (13.6%) compared with 0% before KRG intake. These findings indicate that high frequency of gross deletion in 5'LTR/*gag* and genetic defects prior to HAART are significantly associated with KRG intake and the detection of gross deletion in 5'LTR/*gag* is decreased by HAART.

Keywords: *Panax ginseng*, Korean red ginseng, HIV-1 5'LTR/*gag*, Genetic defects, Highly active antiretroviral therapy, Acquired immuno-deficiency syndrome

INTRODUCTION

Panax ginseng has been used as an herbal medicine for more than 2000 years in the Orient [1]. About 200 constituents from *P. ginseng* have been isolated and characterized [2,3]. Its major components are ginseng saponins and polysaccharides. The major pharmacological effect of ginseng is adaptogenic effect. In other words, ginseng nonspecifically increases resistance to various hazardous stimuli of physical, chemical, and biological stress through immunomodulation and hyphothalmic-pituitary-adrenal axis [4,5]. Recent literatures show the potential of adjuvant or as immunotherapeutics of ginseng [6-14].

Although there have been many reports on the association between genetic defects and clinical courses in HIV-1 infected patients [15-22], gross deletion in the *nef* gene (Δ*nef*) has been reported in very limited proportion even in elite controllers [23]. We previously reported that Korean red ginseng (KRG) increases the frequency of gΔ*nef* and gross deletion in 5'LTR/*gag* regions as well as slow progression in HIV-1 infected individuals [24-28]. Thus, we could obtain several long-term survivors for more than 20 years in the absence of antiretroviral therapy among our ginseng cohort [24,27]. In Korea, the longest survivor among HIV-1 infected patients was diagnosed in 1987 and in the absence of highly active antiretroviral

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therapy (HAART) has maintained his CD4 T cell count since 1994. They all reveal genetic defects in the Δ*nef* or 5'LTR/*gag* although the proportion of defected amplicons was less than 20% and 50%, respectively [27]. In addition, we reported that HAART significantly decreases the frequency of gΔ*nef* caused by KRG intake in 20 hemophiliacs who were infected with Korean subclade B of HIV-1 [26]. In this study, we investigated whether KRG and HAART affect the frequency of gross deletion in 5'LTR/*gag* in 20 hemophiliacs.

MATERIALS AND METHODS

Study subjects

This study consisted of 20 hemophiliac patients (HPs) infected with Korean subclade of HIV-1 subtype B (KSB) [26,29]. All patients were infected with HIV-1 in between late 1989 and April 1991. The time on the first use of domestic clotting factor was described in Table 1 in the reference 29. Age at HIV-1 diagnosis was from 4 to 39 years. Informed written consent was obtained from all subjects before the initiation of the study.

Antiretroviral therapy

As of December 2009, only HP-15 had not received HAART. In addition to two HPs that died (Table 1), 7 HPs (numbers 2, 8, 10, 11, 13, 14, and 16) had not been treated with HAART for the first 10 years after their diagnosis of HIV-1. Thereafter, HPs (numbers 2, 8, 10, 11, 13, 14, and 16) have been treated from April 2006, June 2007, January 2005, November 2003, February 2006, April 2009, and February 2006, respectively (Table 1).

Therapy with Korean red ginseng

Outpatient-based clinical trial of KRG treatment in HIV-1-infected patients was initiated at the Korean National Institute of Health in late 1991. The daily dose of KRG was 5.4 g (six 300 mg capsules three times per day) for men [28,30,31]. KRG has been supplied since November 1991 although the supply of KRG was not consistent before year 2000.

CD4 T cell count and plasma RNA copy number

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

Amplification and sequencing of 5'LTR/*gag*

One-hundred forty seven PBMCs from 20 HPs were obtained between 1991 and 2010. Proviral 5'LTR/*gag* sequences (1,125 bp) were amplified from the PBMC using nested polymerase chain reaction, as previously described [25,27].

Statistical analysis

The data are expressed as the means±one standard deviations (continuous variables) or as counts and percentages (categorical variables). Continuous variables were analyzed by using correlation analyses method; categorical variables were compared with the χ^2 statistics. A p-value of <0.05 were considered statistically significant.

Sequences

The GenBank accession numbers are EF370244-260, EF370362-366, EF370369-372, EU047688-692, and JN613584-804.

RESULTS

Significant correlation between Korean red ginseng intake and decrease in CD4 T cells

CD4 T cell counts were measured during a follow-up period of 117±54 mo prior to HAART. All patients took KRG for various durations. The amount of KRG used prior to the initiation of HAART was 3,625±5,624 g (32 g/mo) for 117±54 mo and the annual decrease in the CD4 T cell count was 53±50/μL (Table 1). When we focused on 13 patients who were followed up for more than 100 mo prior to HAART, there was a significant inverse correlation between amount of monthly KRG intake and annual decrease in CD4 T cells (r=-0.612) (p<0.05).

Number of amplicons

We obtained 436 amplicons for 5'LTR/*gag* genes from 147 PBMCs obtained over 10 yr (range, 3 to 19 yr). Of those, 92 genes (21.1%) from 53 PBMCs (36.1%) showed gross deletion in 5'LTR/*gag*.

Frequency of gross deletion in 5'LTR/*gag* is associated with Korean red ginseng intake

We obtained 30 amplicons at baseline (before KRG intake). Of the 30 amplicons, 3 showed gross deletion

Table 1. Summary of 20 hemophiliac individuals infected with Korean subclade B of HIV-1 subtype B

Patient	Date on diagnosis of HIV-1 infection	Prior to HAART			Plasma RNA copy (/mL) prior to HAART	Amount of KRG (g)	
		Follow-up mo	Annual decrease in CD4+ T	Monthly KRG (g)		Before HAART	During HAART
HP 1	26-11-90	51	28	22	Not tested	1,140	No HAART
HP 2	21-02-91	177	27	10	35,900	1,800	0
HP 3	01-04-91	135	49	15	Not tested	2,000	No HAART
HP 4	24-08-91	111	70	11	8,180	1,210	4,320
HP 5	13-01-92	28	214	21	Not tested	600	No HAART
HP 6	20-01-92	82	72	0	Not tested	0	>5,760
HP 7	22-01-92	64	140	19	20,000	1,200	11,640
HP 8	15-02-92	204	1	74	14,600	>15,390	720
HP 9	25-02-92	171	32	3	23,700	540	180
HP 10	26-03-92	144	28	5	Not tested	780	5,040
HP 11	29-02-92	136	72	1	209,000	180	3,300
HP 12	25-02-92	119	49	0	Not tested	0	1,500
HP 13	29-02-92	163	28	24	4,000	3,900	0
HP 14	19-02-92	204	23	109	4,146	>21,078	1,980
HP 15	16-09-92	123	0	63	111	7,776	No HAART
HP 16	05-12-92	158	22	9	Not tested	1,470	>1,000
HP 17	26-02-93	102	62	0	3,703	0	3,120
HP 18	02-03-93	53	38	145	Not tested	7,680	17,400
HP 19	26-07-93	58	84	0	>10,062	0	9,060
HP 20	04-08-94	51	26	113	12,000	5,760	7,680
Total	Mean±SD	117±54	53±50	32±44	30,485±60,149	3,625±5,624	4,544±4,862

HAART, highly active antiretroviral therapy; KRG, Korean red ginseng; HP, hemophiliac patient.

in 5'LTR/*gag* (10%) (Fig.1). During KRG intake prior to HAART, we obtained 192 amplicons. Of those, 54 showed gross deletion in 5'LTR/*gag* (28.1%, $p<0.05$) (Fig. 1).

Frequency of gross deletion in 5'LTR/*gag* was significantly inhibited during HAART

Among the 20 patients, 16 patients received HAART for 58.5±43.0 mo (range, 4 to 124 mo; median, 66.5 mo). During the HAART period, 12 patients also received KRG. The average amount of KRG and monthly KRG were 4,544±4,862 g (range, 0 to 17,400 g) and 75 g, respectively (Table 1). In regard to HAART, 192 and 214 amplicons were obtained on KRG only and during KRG plus HAART, respectively. Of those, 54 (28.1%) and 35 (16.4%) showed gross deletion in 5'LTR/*gag* for the corresponding periods (Fig. 1). Thus, proportion of gross deletion in 5'LTR/*gag* was significantly inhibited during HAART ($p<0.01$).

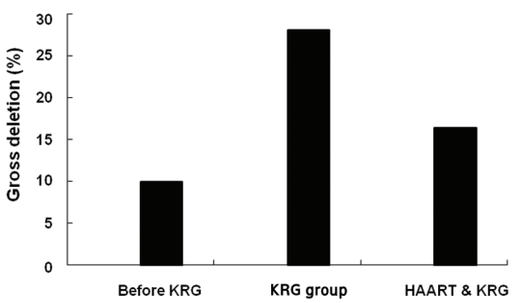

Fig. 1. Changes of the proportion of gross deletion in the 5' LTR/*gag* gene (gΔ). Proportion of gΔ was significantly increased on Korean red ginseng (KRG) intake only (28.1%, 54/192) compared with that prior to KRG intake (10%, 3/30) ($p<0.05$). The detection of gΔ was significantly inhibited during highly active antiretroviral therapy (HAART) and KRG intake compared with during KRG only (16.4%, 35/214) ($p<0.01$).

Nature of gross deletion and a large amplicon

We determined sequences in 82 of 92 gross deletions in 5'LTR/*gag* from 20 HPs and found that the size of the deletions was all different in all patients although in

Table 2. Characteristics of gross deletions in the 5'LTR/*gag* gene

Patient code	Day of sampling	No. of bands by PCR[1)]	No. of PCR reaction with gΔ[2)]	Beginning nt. relative to HIV-1 NL4-3	Size of deletion in 5' LTR (bp)[3)]	Size of deletion in gΔ (bp)	A representative GenBank accession no.
HP-2	21-10-01	1	1	330	459	463	JN613586
	13-09-07	1	1	701	84	0	
	12-06-08	1	1	607	262	144	JN613587
HP-3	29-08-02	2	1	440	349	407	JN613584
HP-4	11-10-02	1	4	484	306	383	JN613588
	27-02-03	1	3	484	306	383	JN613589
	04-01-08	1	2	484	306	383	JN613591
	30-06-08	1	2	484	306	383	JN613593
	22-12-08	1	1	657	132	261	JN613595
HP-5	28-12-94	2	3	579	212	437	6082s
	27-02-95	1	1	627	162	373	6083s
HP-6	17-11-02	1	1	443	Inversion		JN613661
HP-7	20-10-96	1	1	432	358	531	JN613596
		2	1	483	288	0	JN613597
	27-02-97	2	1	350	435	0	JN613598
	07-01-03	2	1	603	186	126	3568s1
HP-8	19-04-95	1	1	657	133	322	EF370245
	15-02-01	1	1	632	158	228	EF370250
	12-12-02	1	3	632	158	478	EF370252
	03-05-04	1	1	110	159	0	EF370256
	06-02-06	1	1	163	8 Plus 17	0	EF370259
	27-09-07	1	3	623			JN613599
	26-04-10	1	1	727	62	457	9198s
HP-9	13-12-02	2	1	Different	ND		
		2	1	625	164	587	JN613600
		2	1	385	406	245	JN613601
		2	1	328	462	110	
	20-11-03	1	1	412	375	36	1651s
	07-09-06	2	1	713	77	480	JN613602
		3	1	722	68	590	JN613603
				342	447	334	JN613604
	19-12-07	2	1	ND			JN613605
		2	2	484	306	5	JN613606
HP-10	30-08-02	1	1	637	153	467	JN613608
		2	1	ND	ND		
		2	1	453	337	271	JN613607
	13-09-07	1	1	639	151	19	JN613609
HP-11	30-10-02	2	1	320	470	153	EF370364
		1	1	304	197	0	EF370366
	09-11-03	1	1	420	370	177	6021
		2	2	613	178	555	JN613610
HP-12	25-10-02	1	1	390	343	0	JN613612
		1	1	602	21	0	JN613711
	09-04-07	1	1	677	113	321	JN613613
		1	1	ND			JN613614
	01-04-09	1	1	635	118	0	6339
	24-08-09	1	1	736	43	0	
	14-05-10	1	1	667	123	310	9197s
HP-14	11-12-96	2	3	ND			
	08-01-03	2	2	635	Rec		3564s
	23-05-07	1	1	ND			
	15-10-09	1	1	371	420	347	
	27-05-10	1	1	667	123	552	JN613621
HP-13	10-03-99	1	1	591	199	605	JN613615
	11-28-02	2	1	424	366	108	JN613616
	03-04-03	2	1	627	163	372	JN613617
		2	1	644	146	533	JN613618
	19-04-07	1	3	354	436	119	JN613619
						89+	
HP-15	08-03-03	2	1	ND			
HP-16	12-09-02	1	1	579	211	362	EU047689
HP-17	03-11-02	2	1				JN613624
	20-02-03	2	2	426	364	21	JN613622
	10-08-04	1	1	671	119	65	JN613759
	16-01-07	1	1		About 200	ND	ND
HP-18	12-04-95	2	2	ND			
	27-04-06	1	1	659	131	505	JN613625
HP-19	07-11-07	1	1	655	135	593	JN613627
HP-20	27-02-03	2	1	567	223	493	JN613628
	08-07-03	1	2	535	255	587	JN613629
	05-06-08	1	1	727[e]	63	210	JN613630
	16-03-10	1	1	669	121	334	JN613631

PCR, polymerase chain reaction; gΔ, *gag* gene; HP, hemophiliac patient; ND, not determined; Rec, recombination.

[1)]1 and 2 denote single short band only and wild type together with short band, respectively.

[2)]No. of PCR product with gΔ out of 4 PCR reactions.

[3)]Base pair.

HP-4, the same size deletions (689 bp) were repeatedly detected (Table 2). Interestingly, there were a case of inversion (HP-5), duplication (EF370252 in HP-8), recombination (HP-14), and insertion (HP-20) together with gross deletion in an amplicon, respectively. Among the 82 amplicons containing gross deletion in 5'LTR/*gag*, only 4 amplicons revealed gross deletion at two sites and 78 amplicons revealed it at one site. All the 82 amplicons revealed gross deletion in 5'LTR region (21 to 470 bp). Ten of the 82 amplicons did not reveal gross deletion in the *gag* region (Table 2). Therefore, the proportion of gross deletion in 5'LTR/*gag* was higher in 5'LTR (82/82) than that in the *gag* region (72/82).

High frequency of nonfunctional genes by G-to-A hypermutations

We determined sequences in 27 amplicons prior to KRG. There was no premature stop codon among the 27 amplicons. We determined sequences for 147 amplicons on KRG only and for 179 amplicons on KRG plus HAART. Maximum number of premature stop codon was 3 in an amplicon. Compared with that prior to KRG therapy (0/27), the amplicons during KRG plus HAART (at 36 codons) revealed significantly higher frequency of stop codons ($p<0.05$). There was also a significant difference between KRG only (at 15 codons) and KRG plus HAART ($p<0.05$). However, when we strictly exclude the amplicons containing gross deletion in the *gag* gene, the number of amplicons prior to KRG, on KRG only, and on KRG plus HAART were 18, 107, and 147, respectively (Table 3). In addition, isoleucine at the initiation codon of Gag protein was detected in six and nine amplicons in KRG only and KRG plus HAART, respectively (Table 3). It was not detected in 18 amplicons before KRG intake. Among the six and nine isoleucines, 5 and 8 were detected together with premature stop codon, respectively. These genetic defects resulted from G-to-A hypermutations (by Hypermut 2.0; Los Alamos National Laboratory, Los Alamos, NM, USA). However, 3 on KRG only and 6 amplicons on KRG plus HAART did not showed genetic defects although they belong to G-to-A hypermutations (Table 3). Differing from the frequency of gross deletion, frequency of G-to-A hypermutations seems higher on KRG plus HAART than KRG only.

Proportion of overall genetic defects

Adding the gross deletions to premature stop codon or isoleucine at initiation codon based on amplicon, the proportions of genetic defects were 10% (3/30) before KRG intake, 33.5% (63/188) on KRG only before HAART and 25.7% (55/214) on KRG plus HAART, respectively. The latter two groups showed significant high proportion of genetic defects compared with data before KRG intake ($p<0.001$ for both) (Fig. 2).

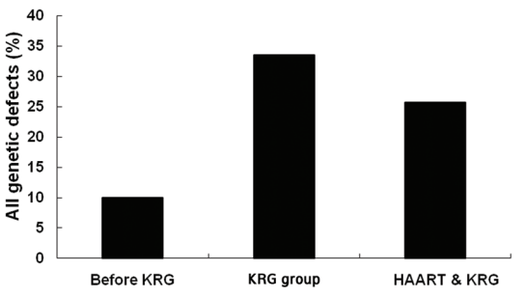

Fig. 2. Changes of the proportion of all genetic defects including gross deletion in the 5' LTR/*gag* gene (gΔ). Proportion of genetic defects significantly increased on Korean red ginseng (KRG) intake only (63/188) and KRG plus highly active antiretroviral therapy (HAART, 55/214) compared with that prior to KRG intake (3/30) (respectively, $p<0.001$) although there was no significant difference between KRG intake only and KRG plus HAART. However, G-to-A hypermutations were higher on KRG plus HAART than KRG only.

Table 3. Frequency of genetic defects in Gag protein

Patient	Prior to KRG intake	On KRG only	On KRG plus HAART	*p*-value[1)]
No. of PCR amplicons	18	107	147	
No. of premature stop codon	0	15	36	< 0.05
No. of I at initiation codon	0	6	9	
No. of total codons (%)	0[2)]	21 (19.6)[2)]	45 (30.6)	< 0.01
No. of PCR amplicon with genetic defects[3)]	0	8 (7.5)	20 (13.6)	
No. of PCR amplicon with G-to-A hypermutations only without genetic defects	0	3	6	
No. of total amplicons with G-to-A hypermutations (%)	0	11 (10.3)	26 (17.7)	

KRG, Korean red ginseng; HAART, highly active antiretroviral therapy; PCR, polymerase chain reaction.
[1)] *p* -value between prior to KRG intake and on KRG plus HAART.
[2)] $p<0.05$ between prior to KRG intake and on KRG only.
[3)] Genetic defects denote premature stop codon and isoleucine at the initiation codon.

Novel insertion, inversion, or duplication in 5'LTR

Novel insertions of 7 bp and 8 bp in 5'LTR were detected in an amplicon in HP-8 (GenBank numbers, EF370257 and EF370258, respectively). Deletion of 9 bp from position 108 and inversion of 609 bp and deletion of 5 bp was observed in seq 3476 in HP-6. A gross deletion and duplication of 105 bp in HP-8 was also observed (GenBank number, EF370252). In HP-13, unusual recombination of terminal 75bp was observed (seq3564s). In HP-20, seq4809 revealed deletion of 273 bp from 727 to 998 and it was replaced with insertion of env gene (367 bp from 8530 to 8896) (Table 2).

DISCUSSION

In this study, we found that frequency of gross deletion in 5'LTR/*gag* is also associated with KRG intake and that the frequency of gross deletion in 5'LTR/*gag* is significantly decreased on KRG plus HAART compared with that prior to HAART. These findings are the same as shown in the frequency of gΔ*nef* in the previous study [26]. This observation may reflect a limit dilution because deleted genes might comprise a minor portion compared with to intact genes in vivo, thus decreasing the chance of detection of gross deletion in 5' LTR/*gag* gene. However, we found a few different findings compared with gΔ*nef* in the same study population. First, there was no difference in the frequency of 5'LTR/*gag* on KRG only between 20 HPs (28.1%) and 3 HPs (-21, 22, and 23) (24.6%; 15/61) who were infected with non-KSB in other countries [12]. In contrast, our previous report showed that the frequency of gΔ*nef* in 20 HPs (20.6%) was 10-fold higher than that (2/100) in 3 HPs (-21, 22, and 23) infected with non-KSB. Taken together, our data suggest that 5'LTR/*gag* region might be more sensitive to KRG intake than in the Δ*nef*. Second, we obtained higher frequency of nonfunctional gene (premature stop codon and I instead of M as initiation codon in Gag protein) (10.3%, 11/107) on KRG intake only. In addition to nonfunctional *gag* gene, there were also frequent G-to-A hypermutations in the *vif* genes (unpublished data). These frequencies in 5'LTR/*gag* and *vif* genes were significantly higher compared to 0.96% (5/522) [26] in the Δ*nef* in the same patients and 0% (0/277) in other patients (p<0.001) [28].

There are many reports on gΔ*nef* reported in many studies. However, there is no report on gross deletion in 5'LTR/*gag* gene together with gΔ*nef*. In detail, Huang *et al*. [32] reported genetic defects in the 5'LTR and *gag* genes of eight long-term survivors. In their work, full viral sequences were obtained. G-to-A hypermutations were seen in only one patient in *gag* gene and 5'LTR. However, there was no gross deletion in the 5'LTR and Δ*nef*. Recently, Calugi *et al*. [33] identified gross deletions in the *env* gene (next to the Δ*nef*). Blankson and Siliciano [34] and Blankson [35] did not detect any gross deletions in full-length sequences of HIV-1 even in elite suppressors, with viral loads of plasma RNA less than 50 copies/mL.

Despite many limitations, this study showed that the frequency of gross deletion in 5'LTR/*gag* is associated with KRG intake. Although further study is needed to determine the source of high frequency of genetic defects in HIV-1 in hemophiliacs, these data provide a new perspective on HIV-1 pathogenesis.

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