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Research Article

Korean Red Ginseng increases defective *pol* gene in peripheral blood mononuclear cells of HIV-1–infected patients; inhibition of its detection during ginseng-based combination therapy

Young Keol Cho [1,*], Jung-Eun Kim [1], Jun-Hee Woo [2]

[1] Department of Microbiology, University of Ulsan College of Medicine, Seoul, Republic of Korea
[2] Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

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ABSTRACT

Background: We have reported that defective *nef* and *gag* genes are induced in HIV-1–infected patients treated with Korean Red Ginseng (KRG).
Methods: To investigate whether KRG treatment and highly active antiretroviral therapy (HAART) affect genetic defects in the *pol* gene, we amplified and sequenced a partial *pol* gene (p-*pol*) containing the integrase portion (1.2 kb) by nested PCR with sequential peripheral blood mononuclear cells over 20 years and compared it with those patients at baseline, in control patients, those taking ginseng-based combination therapy (GCT; KRG plus combinational antiretroviral therapy) and HAART alone. We also compared our findings to look for the full-length *pol* gene (*pol*) (3.0-kb)
Results: Twenty-patients infected with subtype B were treated with KRG for 116 ± 58 months in the absence of HAART. Internal deletion in the *pol* gene (Δ*pol*) was significantly higher in the KRG group (11.9%) than in the control group and at baseline; its detection was significantly inhibited during GCT as much as during HAART. In addition, the Δ*pol* in *p-pol* significantly depended on the duration of KRG treatment. In *pol*, the proportion of Δpol was significantly higher in the KRG group (38.7%) than in the control group, and it was significantly inhibited during GCT and HAART. In contrast, the proportion of stop codon appeared not to be affected by KRG treatment. The PCR success rate was significantly decreased with longer GCT.
Conclusion: The proportion of Δ*pol* depends on template size as well as KRG treatment. HAART decreases the detection of Δ*pol*.

1. Introduction

The accumulation of defective virus in full-length HIV-1 genome level in peripheral blood mononuclear cells (PBMCs) has long been observed in HIV infection [1,2]. These defective viruses reside in circulating PBMCs and in the viral reservoirs as an integrated provirus and result from the high replication rate of HIV-1 and the low fidelity of reverse transcriptase (RT) [3]. This phenomenon has been confirmed by the detection of large internal deletions (IDs) (45.5%) and hypermutated sequences in *gag* (32.4%) among 4.4- to 6.4-kb-sized amplicons [4]. When increasing the targeting size of PCR to 9.1 kb, the proportion of IDs increases to a median of 48.5% depending on the patient [2]. Recently, Bruner et al [5] reported that 98% of proviruses during acute HIV infection were defective and 80% of defective viruses were IDs. Hiener et al [6] identified about 5% of proviruses as intact and potentially replication competent with highly active antiretroviral therapy (HAART). The frequency of intragenomic rearrangements such as IDs, duplications, and inversions is supposed to be high, but remains unknown [7] because all the data have been derived from cloning and sequencing limited numbers of proviruses or their fragments [2]. Physical mapping of defective genomes showed that the frequency of IDs is proportional to their proximity to the central part of the HIV-1 genome, consistent with a deletion mechanism involving a single polymerase jump during reverse transcription [2].

However, the possibility for detection of 1-kb-sized defective genes by conventional PCR is very low. Thus, defective proviral DNA sequences are very rarely detected even in long-term

* Corresponding author. Department of Microbiology, University of Ulsan College of Medicine, 88, Olympic-ro 43-gil, Songpa-gu, Seoul, 05505, Republic of Korea .
E-mail address: ykcho2@amc.seoul.kr (Y.K. Cho).

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nonprogressors (LTNPs) or long-term survivors (LTSs) [8–10]. Since 1995, many studies on LTNPs have been focused on the identification of genetic defects in the *nef* gene [11–13]. Thus, to date, viral genetic defects may not be common in LTNPs/LTSs.

Panax ginseng has been used as a drug for more than 2,000 years in East Asia [14], and recent reports have indicated that ginseng demonstrates immune-modulatory, adjuvant [15,16], and antiviral effects [17]. In late 1991, we began treating HIV-1–infected patients with Korean Red Ginseng (KRG), finding that 6 months of treatment had various beneficial effects, including increases in CD4+ T and CD8+ T cell counts as well as a decrease of the p24 antigen and the soluble CD8 antigen [18–21]. In addition, we have observed a high proportion of genetic defects in HIV-1 in LTSs treated with KRG for a prolonged period [22–24] and also seen clinically significant outcomes seen with more than 25 years of KRG treatment [22,25–32].

To date, only a few reports exist of genetic defects in the *pol* gene by conventional PCR, even in elite controllers or LTNPs [3,33,34].

In this study, we first confirmed that KRG causes defects in the *pol* gene, although it is the most conserved gene of HIV-1 and that the defect rate is significantly affected by the size of the PCR amplicon (about 1.2 kb versus 3.0 kb). This study is the first one which has obtained a high defective gene rate of 11.9% and 38.7% by amplifying the *pol* gene by conventional PCR at around 1.2 kb and 3.0 kb, respectively.

2. Materials and methods

2.1. Study population

We chose patients infected with subtype B who had taken more than three years of KRG in the absence of antiretroviral therapy. In addition, at least five PBMCs should be available for > the 3-year period. Twenty patients were included in the KRG treatment group. Eighteen patients were male, and two were female (Table 1). They all were included in our two previous studies except patient 92-16 [22,31]. Control patients (n = 52) who had not been exposed to KRG or any antiretroviral therapy at the sampling time point were included.

Two hundred fifty-six samples from the KRG treatment group and 57 samples from the control group for p-*pol* gene amplification were used in the present study (Fig. S1). Additional information on these patients, including age, sex, mode of transmission, and year of diagnosis, was shown in Table 1. This study was approved by the institutional review board of Asan Medical Center.

2.2. Therapy with KRG

KRG treatment of HIV-1-infected patients was begun on an outpatient basis at the Korea National Institute of Health in late 1991 [18–24,26–31]. The daily dose of KRG was between 5.4 and 6.0 g for men and 3.0 g for women [35]. Patients took a mean total of KRG (12,519 ± 6,470 g) over the course of 116 ± 58 months with several interruptions. The average monthly dose was 94 ± 31 g (Table 1).

2.3. Amplification of the partial pol and full-length pol genes

Proviral DNA was extracted from uncultured PBMCs, and PCR amplicons were amplified by double-nested (or, rarely, triple-nested) PCR as described elsewhere [29,30,36]. The *pol* gene was amplified via nested PCR with TaKaRa LA-Taq (Takara Bio Inc., Shiga, Japan). First and second PCR reactions were performed in 25 μL and

Table 1
Proportions of internal deletion in the *pol* gene (Δpol) by partial and full-length *pol* PCR (*pol*) in 20 HIV-1 infected individuals treated with Korean Red Ginseng

Patient	Sex/ age	Mode of transmission	Total amount of KRG(g)	Duration of KRG treatment	Monthly KRG (g)	AD	gΔ*nef*	No. of PBMC samples	No. of PBMCs with defects	No. of PCR amplicons	No. of Δpol and other defects
87-05	M9	Blood	21258	254	129	22.3	12.1	11	3	26	8
89-14	M29	Homo	5760	51	132	32.4	30.4	8	2	23	3
89-17	M21	Homo	5076	152	33	17.1	17.5	4	2	21	5 + 1*
90-05	M23	Homo	25602	198	130	7.2	15.8	9	0	30	0
90-18	M28	Homo	13182	147	93	45.0	15.6	7	1	18	1
90-50	M20	Homo	18916	171	111	29.4	18.0	3	1	11	4 + 1(*ins*)
91-20	M30	Homo	14336	188	74	42	15.5	6	1	17	1 + 1*
91-22	M8	Trans	9060	106	98	8.2	8.1	10	3	83	4
92-13	M19	Blood	13470	169	81	12.9	13.5	4	1 + 1(*ins*)	11	2 + 1(*ins*)
92-16	M35	Blood	21078	196	107	22.5	17.2	7	1	20	3
92-48	M47	Homo	8300	72	146	60.8	45.4	10	5	52	9
93-04	M30	Homo	9660	160	60	13.3	4.2	6	1(*ins*)+*	27	0 + 2*,[a] (*ins*)
93-60	M26	Hetero	10710	110	93	26	33.3	4	1 + 1*	19	1 + 2*,[b]
95-87	M32	Homo	4980	40	109	8.5	6.9	8	2	43	5
96-51	M28	Homo	14587	155	88	34.6	11.7	9	2	36	2
97-116	F23	Hetero	9210	162	57	18.1	1.2	13	3	68	3
01-99	F22	Hetero	9738	146	67	11.8	7.0	11	2	62	8
01-179	M37	Homo	8130	82	150	58.8	13.0	11	8	47	17
03-493	M44	Homo	22980	132	174	8.4	11.2 + 100[c]	19	5 + 1(*ins*)	64	8 + 1(*ins*)
04-397	M38	Homo	3120	34	92	79	9.5	4	0	26	0
Total			12,519 ± 6,470	116 ± 58	94 ± 31	27.9 ± 20	15.5 ± 10	164	48	704	84 + 6*+4(*ins*)

Patients 01-99, 93-60, and 97-116 were infected from their husband 87-05, 89-17, and 90-50, respectively
AD, annual decrease of CD4+ T cells before highly active antiretroviral therapy; blood, blood product; Hetero, heterosexual contact; Homo, homosexual contact; *ins*, insertion; KRG, Korean Red Ginseng; PBMC, peripheral blood mononuclear cells; trans, transfusion
[a] Stop codon (*) was detected in an amplicon with the insertion of a nucleotide in patient 93-04 (seq 16337); therefore, total number of amplicons containing defective gene is 93 (13.2%).
[b] Stop codon was detected in an amplicon with internal deletion in patient 93-60 (MH054715).
[c] There was a deletion of 2-bp just following the end of *nef* gene in all amplicons.

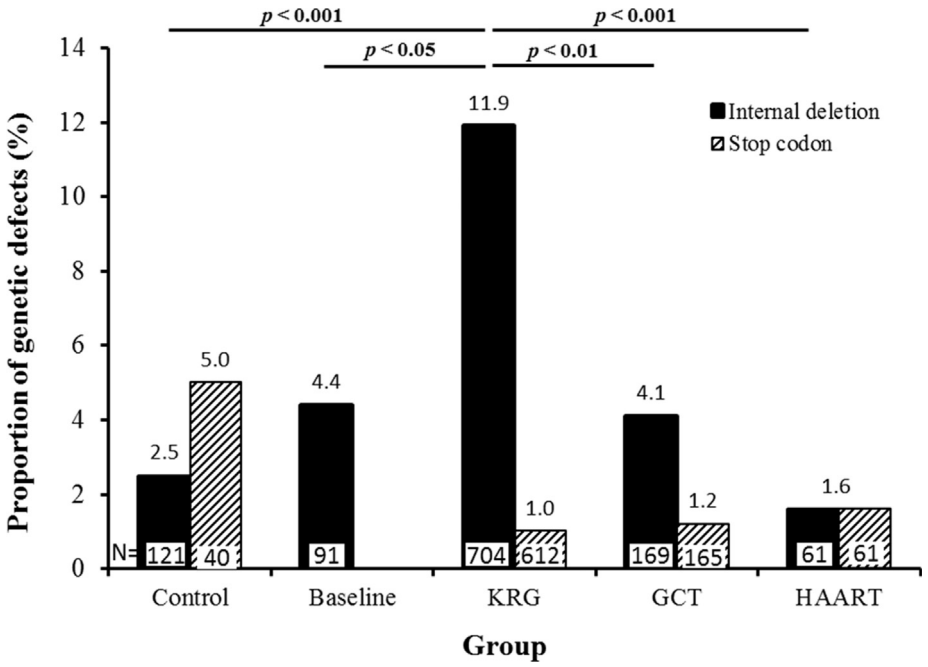


Fig. 1. Effect of Korean Red Ginseng (KRG) treatment on genetic defects in the *pol* gene by partial-length PCR. The proportion of internal deletion in the *pol* gene was significantly higher on KRG than at baseline, in control, and on GCT and HAART. GCT, ginseng-based combination therapy; HAART, highly active antiretroviral therapy.

50 μL reaction mixtures, respectively. The p-*pol* gene (1,232 bp) was amplified using two primer sets, outer primers OBP1 and OBP2 and inner primers OBP3 and OBP4. Rarely, as inner primers, 537 and the OBP4 set was applied (Table S1). The first amplification consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 3 min, followed by a final extension at 72°C for 10 min. The second PCR reaction was performed using 10 μL of the first PCR product, with an amplification protocol consisting of 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min. Reaction volumes were 20 μL for the first PCR and 50 μL for the second PCR. The sequencing primers were OBP3 and 541 (5′-AAGGGGAAGCCATGCA-3′), encompassing nt. 4366 to 4381.

The full-length *pol* PCR amplification (*pol*) used three nested primer sets; the outer set of primers HXB2 and OBP2, OBP2k, and 550; as well as three inner sets of primers were applied: PO1 and OBP4 (3072 bp), P2 and P16 (3,145 bp), and PR3-1 and OBP4 (2858 bp), respectively (Table S1). After initial denaturation at 95°C for 10 min, 38 cycles were run at 95°C for 45 sec, 52°C for 45 sec, and 72 °C for 4 min 50 sec, followed by a final extension step at 72°C for 10

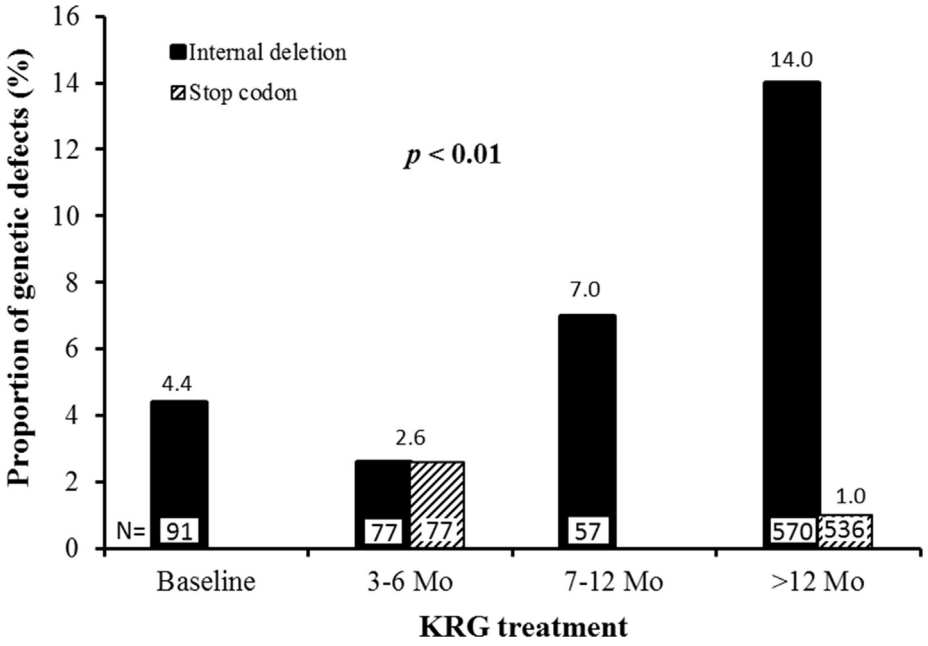


Fig. 2. The proportion of internal deletion in partial *pol* gene (1.2 kb) according to the duration of Korean Red Ginseng (KRG) treatment. It significantly depends on the KRG intake period.

min. The second PCR was performed with 1 μL of the first PCR product. Cycling conditions were as follows: 95°C for 40 sec, 57°C for 40 sec, 72 °C for 4 min 30 sec, and a final extension at 72°C for 10 min. Subsequent amplicons were directly sequenced using Applied Biosystems 3730XL (Thermo Fisher Scientific. Inc., Foster City, CA, USA). We ruled out the possibility of contamination by basic local alignment search tool (BLAST) search compared with sequences from the same patients via our previous GenBank registration [36].

2.4. Statistical analysis

Data were expressed as means ± standard deviations. Statistical significance was estimated by the 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 $p < 0.05$.

2.5. Nucleotide sequences

GenBank accession numbers are JN417005-224, JN561079-084, JN417196-99, KX692369-394, KX782238-39, KX782209-11, KX782015-028, KX692433-449, KX782065-108, MH054579-899, and MN043379-607.

3. Results

3.1. Patient demographics

The clinical characteristics of all patients were described in previous studies [22,31]. They all were infected with HIV-1 subtype B (87-05 and 01-119) including Korean subclade B (n = 18). All individuals belonged to the long-term slow progressor group except for 4 patients (89-14, 92-48, 01-179, and 04-397) (Table 1, Fig. S1). Patient 92-16 is a long-term slow progressor with hemophilia infected with HIV-1 in 1991 with a contaminated clotting factor 9, as in patient 92-13. Despite consistent KRG treatment, his CD4+ T cell gradually decreased over 17 years.

3.2. The relationship between KRG consumption and internal deletions in the pol gene

We amplified the p-*pol* gene with 164 samples on KRG treatment. Of those, 48 revealed genetic defects (29.3%) consisting of 44 IDs and 4 insertions or stop codons (SCs).

In amplicons of the p-*pol* gene, the deletion site spanned the end of RT and the integrase site at two-thirds. Of the 121 and 91 amplicons obtained in control patients and at baseline, three amplicons (2.5%) in patients 89-14 and 92-23, and four amplicons (4.4%) in 3 patients (89-14, 92-13, and 93-04) were grossly deleted, whereas 84 (11.9%) of the 704 amplicons on KRG treatment were grossly deleted ($p < 0.001$ and $p < 0.05$) (Table 1 and Fig. 3). In the 11 amplicons containing Δ*pol*, the exact deletion junction could not be identified, most likely because the deletions encompassed binding sites for primers used in the nested PCRs. A Δ*pol* contained also premature SCs in patient 93-04. We divided the 704 amplicons into three groups by 3-6 months, 7-<12 months, and >12 months. The proportions of Δ*pol* amplicons were in order, 2.6% (2/77), 7.0% (4/57), and 14.0% (80/570) ($p < 0.01$) (Fig. 2). There were a few duplications (JN417155) and inversions.

From the control patients, 121 amplicons were obtained. Among this group, three were grossly deleted ones (2.5%). In the KRG-treated group, 704 amplicons were obtained. Of them, 93 amplicons (13.2%) including 6 SCs and 4 insertions contained a defective gene (Table 1). Results were significantly higher than 4.4% at baseline ($p < 0.05$) and 2.5% in control patients ($p < 0.001$; Fig. 1).

Seventeen patients exhibited Δ*pol* on KRG treatment, and the deletion junction could not be defined in patient 92-16 (Table 1, Fig. 3). The locations and sizes of IDs are described in Fig. 3. All Δ*pol* were larger than 200 bp and observed at one site in an amplicon with the exception in patient 95-87; ID occurred at two sites (28 bp from 4741 to 4768 and 19 bp from 4810 to 4828) (JN417079) in the same 4 double bands (Fig. 3). The same size ID of 987 bp at the same location was observed in 13 amplicons in 4 patients (92-48, 96-51, 01-179, and 03-493) on KRG and each one at baseline, control, and GCT (Fig. 3). We confirmed that those were not contaminated amplicons. Even if they were assumed to be due to contamination and excluded, the proportion of Δ*pol* was significantly higher on KRG than in all other groups including the control group in Fig. 1 (1.7%) ($p < 0.05$). However, 3 patients (90-05, 93-04, and 04-397) did not reveal any ID (Table 1), although they all revealed gross deletions in the *nef* gene (gΔ*nef*) of 4.2%-17.2% on KRG treatment (Table 1 and Fig. S1).

3.3. Effect of HAART on the detection of Δpol and SC

Detection of Δ*pol* in p-*pol* was significantly inhibited in the period of GCT receiving KRG plus HAART (4.1% as of 4/169) compared with the KRG-only period (11.9%) ($p < 0.01$) (Fig. 1).

The same phenomenon was obtained by *pol* PCR, and it will be described later.

3.4. No association between KRG treatment and SC

Of the 612 p-*pol* amplicons on KRG (excluding 92 obtained by RT-PCR), 6 amplicons from 4 patients revealed SC (1.0%). There was no SC in 46 amplicons at baseline (0%). SC in the control group (6.7%) was slightly significantly higher than in the KRG group ($p = 0.053$).

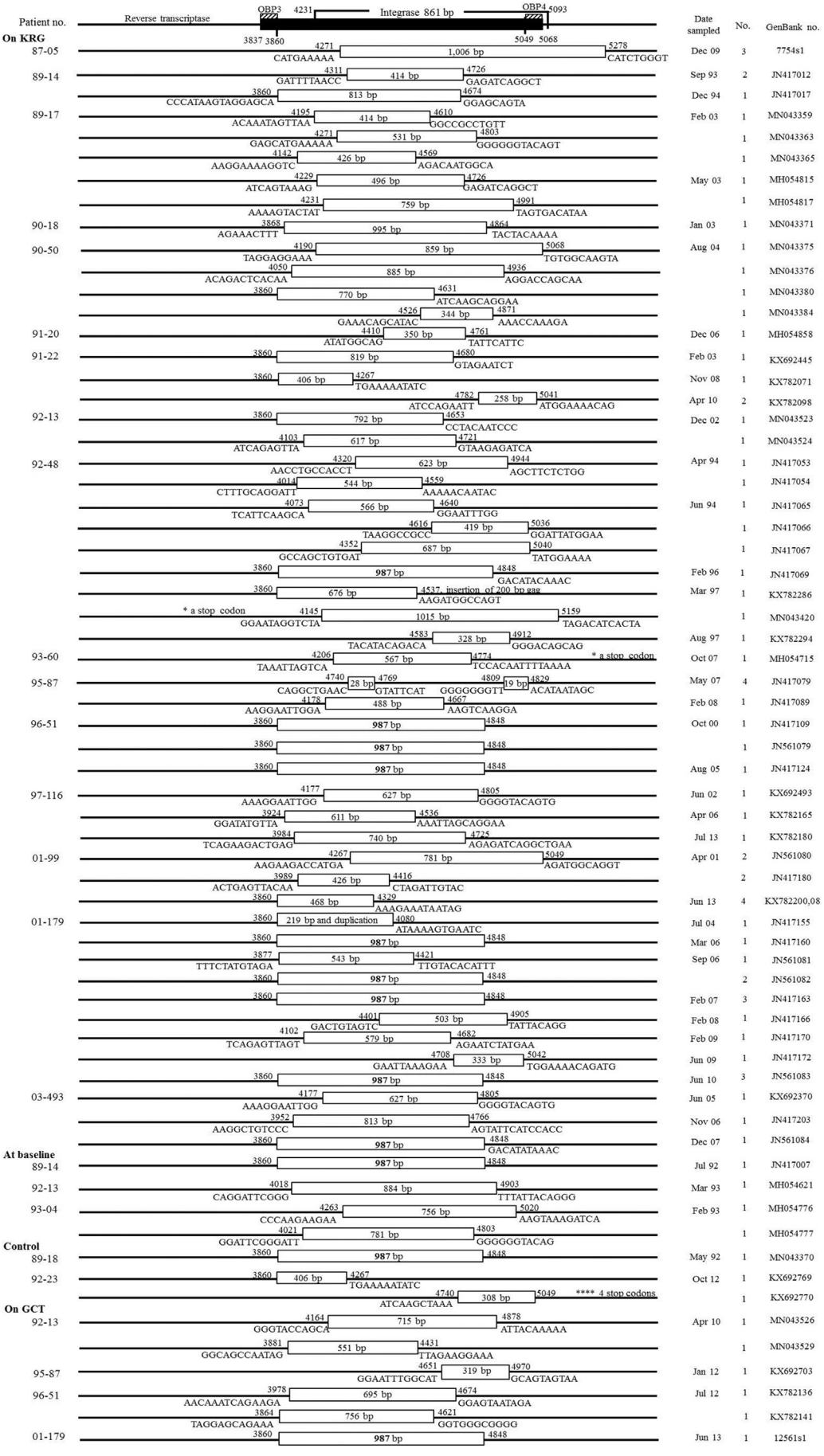
In the control group, two amplicons (5%) from patient 92-23 revealed SCs. Two (1.2%) of 165 amplicons on GCT and one of the 61 amplicons on HAART only (1.6%) revealed SCs. These results are almost the same as those in the *nef* gene (0.9% on KRG and 0.6% on GCT) [24]. Taken together, the data suggest that the induction of SC was not affected by KRG treatment.

3.5. A higher proportion of Δpol by full-length pol PCR

We also performed full-length *pol* PCR to determine whether the frequency of Δ*pol* was affected by the PCR target size in the same patients. As a total, 383 by *pol* PCR were amplified and sequenced.

We obtained 93 PCR amplicons on KRG treatment. Of those, 36 revealed IDs (38.7% as of 36/93). The proportion of SC was 1.1% (1/93). In the *pol* PCR, the proportion of Δ*pol* was also significantly higher in the KRG-treated group (38.7%) than 17% (17/100) in the control group ($p < 0.0001$), and however, its detection was also significantly inhibited during GCT as shown in the aforementioned p-*pol* (Fig. 4). In the *pol* PCR, the Δ*pol* was also significantly higher in the control group (6.8-fold; $p < 0.05$) compared with p-*pol* PCR (Fig. 4). The proportion of SC was mildly higher in GCT (6.3%) than 1.1% in the KRG period (1/93) ($p = 0.09$). Interestingly, there was a similar frequency between GCT and HAART alone, suggesting that SC might be affected not by KRG, but by HAART.

The locations and sizes of IDs are described in Fig. S2. All Δ*pol* were larger than 830 bp and observed at one site in an amplicon with 3 exceptions (Fig. S2). The ID of 2,277 bp at the same location was observed in 20 amplicons (55.5%) in 6 patients on KRG and two in control (Fig. S2). We confirmed that they are not contaminated. Even if they were assumed to be due to contamination and excluded in statistics, the proportion of Δ*pol* was


Patient no.
Reverse transcriptase
OBP3 4231
Integrase 861 bp
OBP4 5093
3837 3860
5049 5068
Date sampled
No.
GenBank no.
On KRG
87-05 CATGAAAAA 4271 1,006 bp 5278 CATCTGGGT Dec 09 3 7754s1
89-14 GATTTTAACC 4311 414 bp 4726 GAGATCAGGCT Sep 93 2 JN417012
CCCATAAGTAGGAGCA 3860 813 bp 4674 GGAGCAGTA Dec 94 1 JN417017
89-17 ACAAATAGTTAA 4195 414 bp 4610 GGCCGCCTGTT Feb 03 1 MN043359
GAGCATGAAAAA 4271 531 bp 4803 GGGGGGTACAGT 1 MN043363
AAGGAAAAGGTC 4142 426 bp 4569 AGACAATGGCA 1 MN043365
ATCAGTAAAG 4229 496 bp 4726 GAGATCAGGCT May 03 1 MH054815
AAAAGTACTAT 4231 759 bp 4991 TAGTGACATAA 1 MH054817
90-18 AGAAACTTT 3868 995 bp 4864 TACTACAAAA Jan 03 1 MN043371
90-50 TAGGAGGAAA 4190 859 bp 5068 TGTGGCAAGTA Aug 04 1 MN043375
ACAGACTCACAA 4050 885 bp 4936 AGGACCAGCAA 1 MN043376
3860 770 bp 4631 ATCAAGCAGGAA 1 MN043380
GAAACAGCATAC 4526 344 bp 4871 AAACCAAAGA 1 MN043384
91-20 ATATGGCAG 4410 350 bp 4761 TATTCATTC Dec 06 1 MH054858
91-22 3860 819 bp 4680 GTAGAATCT Feb 03 1 KX692445
3860 406 bp 4267 TGAAAAATATC Nov 08 1 KX782071
ATCCAGAATT 4782 258 bp 5041 ATGGAAAACAG Apr 10 2 KX782098
92-13 3860 792 bp 4653 CCTACAATCCC Dec 02 1 MN043523
ATCAGAGTTA 4103 617 bp 4721 GTAAGAGATCA 1 MN043524
92-48 AACCTGCCACCT 4320 623 bp 4944 AGCTTCTCTGG Apr 94 1 JN417053
CTTTGCAGGATT 4014 544 bp 4559 AAAAACAATAC 1 JN417054
TCATTCAAGCA 4073 566 bp 4640 GGAATTTGG Jun 94 1 JN417065
TAAGGCCGCC 4616 419 bp 5036 GGATTATGGAA 1 JN417066
GCCAGCTGTGAT 4352 687 bp 5040 TATGGAAAA 1 JN417067
3860 987 bp 4848 GACATACAAAC Feb 96 1 JN417069
3860 676 bp 4537, insertion of 200 bp gag AAGATGGCCAGT Mar 97 1 KX782286
* a stop codon GGAATAGGTCTA 4145 1015 bp 5159 TAGACATCACTA 1 MN043420
TACATACAGACA 4583 328 bp 4912 GGGACAGCAG Aug 97 1 KX782294
93-60 TAAATTAGTCA 4206 567 bp 4774 TCCACAATTTTAAAA * a stop codon Oct 07 1 MH054715
95-87 CAGGCTGAAC 4740 28 bp 4769 GTATTCAT 4809 19 bp 4829 GGGGGGGTT ACATAATAGC May 07 4 JN417079
AAGGAATTGGA 4178 488 bp 4667 AAGTCAAGGA Feb 08 1 JN417089
96-51 3860 987 bp 4848 Oct 00 1 JN417109
3860 987 bp 4848 1 JN561079
3860 987 bp 4848 Aug 05 1 JN417124
97-116 AAAGGAATTGG 4177 627 bp 4805 GGGGTACAGTG Jun 02 1 KX692493
GGATATGTTA 3924 611 bp 4536 AAATTAGCAGGAA Apr 06 1 KX782165
TCAGAAGACTGAG 3984 740 bp 4725 AGAGATCAGGCTGAA Jul 13 1 KX782180
01-99 AAGAAGACCATGA 4267 781 bp 5049 AGATGGCAGGT Apr 01 2 JN561080
ACTGAGTTACAA 3989 426 bp 4416 CTAGATTGTAC 2 JN417180
3860 468 bp 4329 AAAGAAATAATAG Jun 13 4 KX782200,08
01-179 3860 219 bp and duplication 4080 ATAAAAGTGAATC Jul 04 1 JN417155
3860 987 bp 4848 Mar 06 1 JN417160
TTTCTATGTAGA 3877 543 bp 4421 TTGTACACATTT Sep 06 1 JN561081
3860 987 bp 4848 2 JN561082
3860 987 bp 4848 Feb 07 3 JN417163
GACTGTAGTC 4401 503 bp 4905 TATTACAGG Feb 08 1 JN417166
TCAGAGTTAGT 4102 579 bp 4682 AGAATCTATGAA Feb 09 1 JN417170
GAATTAAAGAA 4708 333 bp 5042 TGGAAAACAGATG Jun 09 1 JN417172
3860 987 bp 4848 Jun 10 3 JN561083
03-493 AAAGGAATTGG 4177 627 bp 4805 GGGGTACAGTG Jun 05 1 KX692370
AAGGCTGTCCC 3952 813 bp 4766 AGTATTCATCCACC Nov 06 1 JN417203
3860 987 bp 4848 GACATATAAAC Dec 07 1 JN561084
At baseline
89-14 3860 987 bp 4848 Jul 92 1 JN417007
92-13 CAGGATTCGGG 4018 884 bp 4903 TTTATTACAGGG Mar 93 1 MH054621
93-04 CCCAAGAAGAA 4263 756 bp 5020 AAGTAAAGATCA Feb 93 1 MH054776
GGATTCGGGATT 4021 781 bp 4803 GGGGGGTACAG 1 MH054777
Control
89-18 3860 987 bp 4848 May 92 1 MN043370
92-23 3860 406 bp 4267 TGAAAAATATC Oct 12 1 KX692769
ATCAAGCTAAA 4740 308 bp 5049 **** 4 stop codons 1 KX692770
On GCT
92-13 GGGTACCAGCA 4164 715 bp 4878 ATTACAAAAA Apr 10 1 MN043526
GGCAGCCAATAG 3881 551 bp 4431 TTAGAAGGAAA 1 MN043529
95-87 GGAATTTGGCAT 4651 319 bp 4970 GCAGTAGTAA Jan 12 1 KX692703
96-51 AACAAATCAGAAGA 3978 695 bp 4674 GGAGTAATAGA Jul 12 1 KX782136
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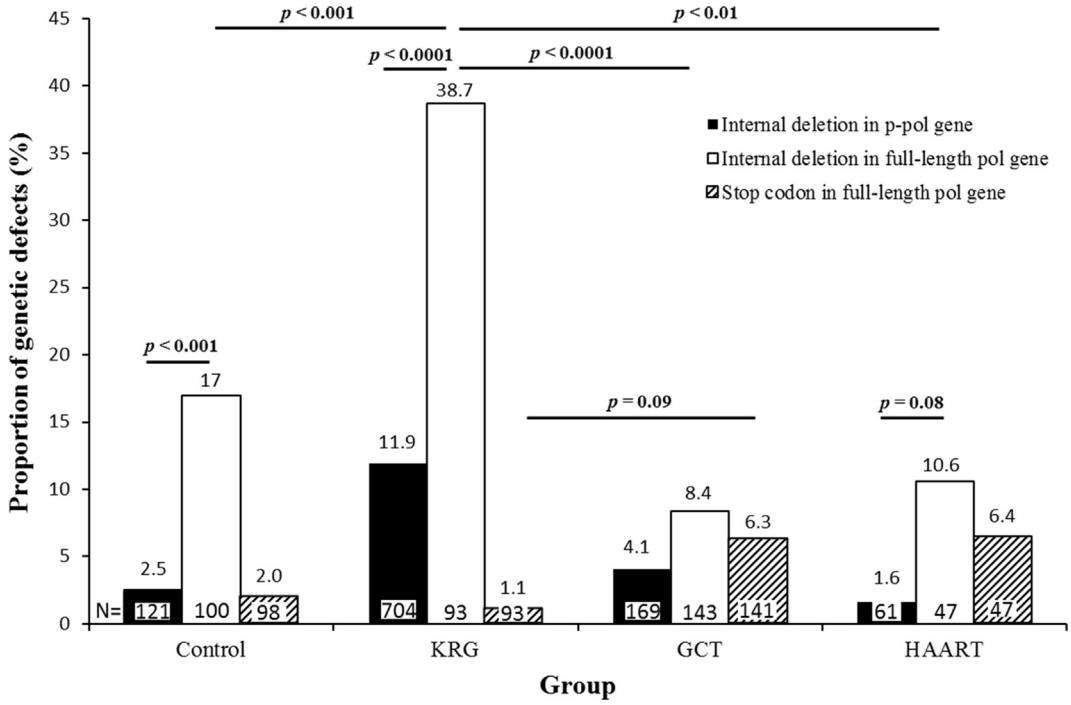

Fig. 4. Comparison of the proportion of genetic defects by Korean Red Ginseng (KRG) between partial *pol* gene covering integrase portion (p-*pol*; 1.2-kb) and full-length *pol* gene amplifications (*pol*; 3.0-kb). In the case of p-*pol* amplicons, the proportion of internal deletion only was shown. As a total, 383 *pol* genes were amplified and sequenced. In the *pol* amplicons, the proportion of internal deletion was significantly higher in the KRG-treated group than in the control group ($p < 0.001$), and however, its detection was significantly inhibited during GCT as shown in p-*pol* above ($p < 0.0001$). The proportion of internal deletion in the full-length amplification was significantly higher in two groups of control and KRG than in p-*pol* gene. The proportion of premature stop codon (SC) was mildly higher in GCT (6.3%) than 1.1% in KRG ($p = 0.094$), suggesting that SC was not associated with KRG treatment in *pol* amplicons. In GCT, the proportion of SC was also significantly higher in *pol* amplifications (6.3% as of 9/141) than 1.2% (2/165) in the p-*pol* gene ($p < 0.05$). However, there was no difference in the frequency of internal deletion and SC between GCT and HAART. Even when internal deletion and SC in *pol* gene was combined, the proportion of defective genes was also significantly higher in the KRG group than in GCT ($p < 0.01$). GCT, ginseng-based combination therapy; HAART, highly active antiretroviral therapy.

significantly higher in KRG than in all other groups (data not shown).

3.6. The longer the PCR target size, the higher the failure rate

As expected, the defective gene rate was significantly higher as the size of the target gene of the PCR increased and the defective rate was significantly increased at the 3.0-kb *pol* than in the 1.2-kb p-*pol* PCR (Fig. 4).

In this study, we also found that the success rate of PCR amplification by full-length *pol* PCR was gradually decreased as the GCT period became longer, regardless of the type of second primer sets. Briefly, after 6 years of ART, it decreased from 63% at baseline to 29% (Fig. S3). It is thought this is caused by an increase in the frequency of the SC and the deficiency of the provirus.

4. Discussion

In this study, we report that Δ*pol* can be induced by taking KRG, although the *pol* gene of HIV-1 is the most conserved gene and that the proportion of Δ*pol* increases with a longer target size of PCR. In both 1.2-kb and 3.0-kb targeting PCR, the Δ*pol* was significantly lower during GCT and HAART than in the KRG treatment period because of inhibited detection by the limit dilution effect. In regard to SCs, such association was not observed in the *pol* and *nef* (n = 140) genes [31], although there was an association between KRG treatment and in the *gag* gene [32]. Nevertheless, the proportion of Δ*pol* in the p-*pol* gene (1,232-bp) (11.9%) was lower than gΔ*nef* (14.7%) with smaller target size (771-bp), suggesting that the *pol* gene is more conserved than the *nef* gene [24].

The accumulation of cell-associated defective viruses is generally the consequence of frequent sequential passages of the virus at high multiplicities of infection and of the substantially longer lifetime of nonproductively infected cells than productively infected cells [2]. Production of large amounts of HIV-1 particles and rapid turnover of HIV-1–infected cells and virions form a natural base for the appearance of defective HIV-1 genomes. A high local multiplicity of infection in lymphoreticular tissue is where the most virus replicates; the immune system rapidly depletes productively infected cells [37,38] and selects cells infected with defective viruses that do not express the HIV-1 genome. The occurrence of defective retroviruses is related to the frequency of point mutations, ranging from 10^{-5} to 10^{-4} per nucleotide per cycle, and of intragenomic rearrangements [7]. In addition, HAART itself also affects the mutations and their detection. For example, before initiation of HAART, the frequency of full-length proviruses has been reported to be 36% at

Fig. 3. Position and size of internal deletions in the *pol* gene by partial *pol* gene amplification. Sequences are numbered according to the NL43 reference clone. Sixty-nine, 4, 3, and 6 Δ*pol* were depicted on the KRG group, at baseline, in control, and on GCT, respectively. For 10 amplicons containing Δ*pol* in patient 03-493, the exact deletion junction could not be identified. The size of deletion ranges from 219 bp to 987 bp except for two extremes; 47-bp and 1008-bp. The same size Δ987 bp at the same location was observed in 13 amplicons in 4 patients (92-48, 96-51, 01-179, and 03-493) on KRG and each one at baseline, control, and GCT. In this case, the site of deletion began just after the forward primer (OBP3) binding site, and the primer OBP3 sequence was once described in patient 89-14 for clarity of the figure. The same deletions of 406-bp were detected in patient 91-22 on KRG and control patient 92-23. Short sequence repeats were identified at some deletion junction as shown in reference 4. GCT, ginseng-based combination therapy.

high virus concentrations; six years later and 3 years after achieving a plasma viral load <50 copies/mL, this percentage had decreased to 6% [39]. Our data also showed that PCR amplification (3.2-kb) itself was significantly affected by the duration of HAART (Fig. S3).

In PCR, length-dependence problems can arise when amplifying differently sized alleles, pseudogenes, or heterogeneous genomes involving deletions [40]. We have also confirmed this finding in the 5′ LTR/gag gene. In other words, when the full-length *gag* gene was amplified, the ID was significantly higher (168/617) than that (30/59) when amplifying the partial length *gag* (about 1200-bp) in KRG-treated patients (27.2% vs 50.8%; $p = 0.0001$) (data not shown).

We previously reported a strong correlation between KRG treatment and g*Δnef* ($r = 0.89$, $p < 0.01$) [31] as well as the high proportion of gross deletion in the 5′ LTR/gag [29,32]. In the present study, we have extended these findings in the *pol* gene.

To date, approximately 200 substances, including ginsenoside, polysaccharides, polyacetylenes, peptides, and amino acids, have been isolated from Korean ginseng [41]. In particular, triterpenoid saponins have antiretroviral effects [42]. For example, several ginsenosides, including polyacetylene ginsenoside-Ro from *P. ginseng* and xylanase from *Panax notoginseng*, were found to inhibit the replication of HIV-1 [43–45], and the ginsenosides Rb1, Rb2, Rb3, and Rc inhibit HIV replication in vitro (patent no, CN1745756A).

KRG-induced genetic changes in HIV-1 may indirectly result from innate enhancement rather than adaptive immunity [46–48], immune modulation toward Th1-cytokines [49,50], anti-inflammatory response through TLR4-induced NF-*k*B [51]; it may also result from the attenuation of hyperimmune activation state, as shown in the decrease of soluble CD8 antigen [19,28]. The mechanism of action of KRG is more likely to be explained as "indirect ravage due to comprehensive immunological pressure or compression on HIV-1″ than the effect of latency-reversing agents, expressed as “shock and kill”.

As mentioned previously, the efficacy of KRG on the HIV-1 gene is not only nonspecific but also includes more than 200 active constituents. Thus, it is difficult to mention the mechanism immediately.

Therefore, further studies are needed to elucidate the mechanism by which KRG induces IDs in HIV-1 genes.

To my knowledge, this report is the first documented study on the gross deletion or ID in the *pol* gene level by a specific treatment. Recently, a significant proportion of the resulting proviruses have revealed large deletions when recombination was blocked [52]. The use of KRG induces nonspecific defects in the HIV-1 gene, such as 5′LTR/*gag*, *nef*, and *pol* genes, leading to a higher percentage of a defective provirus. At the same time, complete inhibition of virus replication by HAART suggests that fewer transcriptionally active proviruses, as well as a decrease of DNA [53], and ultimately provirus, can be removed. Therefore, concurrent therapy with KRG and HAART as GCT could be an ideal regimen similar to artemisinin-based combination therapy in malaria therapy [54]. Because the incidence of intact provirus in patients on HAART is reported to be 5% [6], we can presume that the incidence of intact provirus is expected to be lower in patients undergoing GCT, considering that such IDs occur throughout the entire genome as well as the *pol* gene during the administration of KRG. Finally, it can be presumed that after a long GCT, it will be closer to 0%.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix 1. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.05.011.

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