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

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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...
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 > 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 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-Tag (Takara Bio Inc., Shiga, Japan). First and second PCR reactions were performed in 25 µL and

| Patient | Sex/age | Mode of transmission | Total amount of KRG(g) | Duration of KRG treatment | Monthly KRG(g) | AD | gΔ nef | No. of PCR amplicons | No. of PBMCs with defects | No. of PBMCs | No. of PCR amplicons with 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 | 9 | 0 | 30 | 0 |
| 90-18 M16 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 Momo 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* | |
| 93-60 M26 Hetero 10710 | 110 | 93 | 26 | 33.3 | 4 | 1 + 1* | 19 | 1 + 2* |
| 95-87 M32 Hetero 4980 | 100 | 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- F23 Homo 82 | 162 | 57 | 18.1 | 1.2 | 13 | 3 | 68 | 3 |
| 116 01-99 M23 M27 Hetero 9738 | 146 | 67 | 11.8 | 7.0 | 11 | 2 | 62 | 8 |
| 01-90 M37 Homo 8130 | 82 | 150 | 58.8 | 13.0 | 11 | 8 | 47 | 17 |
| 179 04- M44 Homo 22980 | 132 | 174 | 8.4 | 11.2 + 100 | 19 | 5 + 1/(ins) | 64 | 8 + 1/(ins) |
| 397 04- 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, transfection

* 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.
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.

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. 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.

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 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. 1). Patient 92-16 is a long-term slow progressor with hemophilia infected with HIV-1 in 1991 with a contaminated clotting factor, as in patient 92-13. Despite consistent KRG treatment, his CD4+ T cell gradually decreased over 17 years.

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

Detection of Δpol 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%) (\( p < 0.05 \) (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 Δpol 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 Δpol 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 PCR (Fig. 4). In the pol PCR, the Δpol 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 Δpol 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 Δpol was Seventeen patients exhibited Δpol 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 Δpol 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 one each 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 Δpol 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 (Δgαef) of 4.2%-17.2% on KRG treatment (Table 1 and Fig. S1).
defective genes was also significant in the KRG group than in the control group (p < 0.001). 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 p-pol amplifications. In GCT, the proportion of SC was also significantly higher in full-length 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 p-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.

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 Δpol can be induced by taking KRG, although the pol gene of HIV-1 is the most conserved gene and that the proportion of Δpol increases with a longer target size of PCR. In both 1.2-kb and 3.0-kb targeting PCR, the Δpol 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 Δpol in the p-pol gene (1,232-bp) (11.9%) was lower than Δnef (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

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**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 Δpol were depicted on the KRG group, at baseline, in control, and on GCT, respectively. For 10 amplicons containing Δpol 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 Δ deletion 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 OBF3 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.

**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.
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 gag nef (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. CN1745796A). 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-κB [51]; it may result from the attenuation of hyperimmunological 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 artesiminin-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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