Investigation of KIT Gene Polymorphisms in Korean Cattle

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ABSTRACT

KIT gene is the major causative gene for coat color variation in diverse animal species. This gene regulates melanocyte migration from the neural crest to target tissues and the mutation of this gene can affect dominant white phenotypes in animals. Because this gene has a major influence for the coat color variation, single nucleotide polymorphisms (SNPs) in 14 Korean cattle (Hanwoo) and 5 Holstein individuals were investigated. The Hanwoo DNA samples included three different colored (5 Black, 5 Yellow and 4 Stripe) animals. Total 126 polymorphisms have been identified and 23 of them are located in the exon region. Also, 5 bp (TTCTC) and 3 bp (TCT) intronic indels in intron 3 and intron 5, respectively, were identified. Out of 23 exonic polymorphisms, 15 SNPs are the missense mutations and the rest of the SNPs are silence mutations. The neighbor-joining phylogenetic tree was constructed for the different colored animals using the obtained KIT gene sequences. Holstein breed showed a clear breed-specific cluster in the phylogenetic tree which is differed from Hanwoo. Also, three colored Hanwoo animals were not discriminated among the breeds. The KIT gene polymorphisms identified in this study will possibly give some solutions for the color variations in cattle with further verifications.

(**Key words** : Coat color, KIT gene, Korean cattle, Phylogenetic analysis)

INTRODUCTION

In terms of the coat color, KIT gene has an important role for regulation of melanocyte migration from the neural crest to target tissues and the mutations in this gene can affect the coat color variations in diverse animal species. The process of melanogenesis is involved in motivating the pigment synthesis on the dorsolateral pathway to deposit the final destination in the skin (Besmer et al., 1993). Also, particular mutations in the KIT gene have been reported in myeloproliferative disorders, mast cell neoplasms, seminoma and acute myeloid leukemia in human gastrointestinal stromal tumors (Nagata et al., 1995; Nakata et al., 1995; Gari et al., 1999; Tian et al., 1999). In human and mouse, this gene is known to affect the pigmentation defects (Chabot et al., 1988; Fleischman et al., 1991; Geissler et al., 1988; Spritz et al., 1992). Also, the polymorphisms in KIT gene can cause different pigmentation patterns and coat color in horse and pig (Brooks and Bailey, 2005; Haase et al., 2007; Giuffra et al., 1999; Marklund et al., 1998, 1999; Pielberg et al.,

2002). Therefore, for the breed formation in animals, selection and fixation of breed specific coat colors have been performed, which can also possibly fix the KIT alleles.

In cattle, the white spotting locus has been mapped on bovine chromosome 6 (BTA6) near the KIT gene in a Hereford-cross population. In this region, a QTL has been mapped in German Holstein-Friesian, Simmental and Holstein-Friesian × Jersey cross (Grosz and MacNeil, 1999; Liu et al., 2009; Reinsch et al., 1999). Recently, 111 polymorphisms from 18 individuals, from three breeds (Angus, Hereford and Holstein-Friesian), have been identified in the bovine KIT gene and 28 haplotypes were identified (Fontanesi et al., 2010). Also, they were considered carriers of putative alleles at the spotting locus for their coat color patterns, which can indicate selection signature in this region. Based on the sequencing results from seven cattle breeds, two single nucleotide polymorphisms (SNPs), one in exon 2 (g.72779776C > T) and the other in exon 3 (g.72783182A > G) of KIT gene, were identified as candidate markers for different coat color patterns (Fontanesi et al., 2010). In the discriminatory power,

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Italian Simmental was almost fixed for the two common alleles (g.72779776C and g.72783182A) and two haplotypes have been discriminated in spotted breeds.

The Korean cattle, named as Hanwoo, have three different coat colors including black, yellow and stripe. Identification of KIT gene polymorphisms in three different colored Hanwoo and white spotting Holstein breed have been investigated in this study, which can possibly give the solutions for the coat color variations in Hanwoo.

MATERIALS AND METHODS

1. Experimental animals

Based on the coat color patterns, three different Hanwoo breeds (black, stripe, yellow) have been used in this experiment. Also, as a control breed, Holstein was used. Five blood samples in each breed except stripe Hanwoo (four samples) were collected from National Institute of Animal Science (NIAS). Genomic DNAs were extracted from blood samples using PrimePrep™ Genomic DNA Isolation Kit (GeNet Bio, Korea) according to the manufacturer's instructions.

2. PCR amplification and sequencing

Fifteen primer sets were designed which cover the part of flanking region and intronic location with all 21 exon in the KIT gene reported by Fontanesi et al. (2009) (Table 1). PCR

reactions included approximately 100 ng of genomic DNA, 2.5 µL of 10X buffer [Tris-HCl (pH 9.0), PCR enhancers, (NH₄)₂SO₄, 20 mM MgCl₂], 2.0 µL of 10 mM dNTPs mixture (2.5 mM each of dATP, dCTP, dGTP and dTTP), 1 µL of 10 pM of each primer and 1 U *HS Prime Taq* (GeNet Bio, Korea) in a 25 µL reaction volume. PCR was performed in a My-Genie96 Thermal Block (Bioneer) with an initial denaturation step at 94°C for 5 min followed by 35~40 cycles of 30 sec at 94°C, 30~40 sec at 60°C, 40 sec ~2 min at 72°C and a final extension step at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gels with ethidium bromide and DNA bands were visualized under ultraviolet light. Purification of PCR products was performed using an Accuprep® PCR purification kit (Bioneer) according to the manufacturer's instructions. Purified PCR products were also confirmed using agarose gels for sequencing. All the purified PCR products were sequenced by Genotech (www.genotech.co.kr).

3. Sequencing analysis

The cattle KIT gene sequences were aligned using the ClustalW program (Thompson et al., 1994) and saved as the bioedit format. Nucleotide substitution positions were confirmed in National Center for Biotechnology Information (NCBI) data base (Btau_4.2) by using MEGA5 software (Tamura et al., 2011). Also, neighbor-joining phylogenetic tree using Rectangular Kimura 2-parameter model with 1000 bootstrap replications was constructed. Furthermore, SNP

Table 1. Primers used in this study for amplifying all exons in bovine KIT gene

Primer no.	Forward Primer (5' - 3')	Reverse Primer (3' - 5')	Amplified exon	Size (bp)
1	CCGGAACGTGGAACAGAG	AAGTTGTGCGTGAGAACCG	Exon-1	533
2	GCAAATCTCCGTAGGCATTAGG	ATACCCGAAGCCACTATGCATC	Exon-2	1094
3	GAATAAAGTCGGCAGCCATTG	CAATAGCTAAATGCCCCTGGT	Exon-3	1140
4	GGAAGCCTTACACTGTTTGAA	GAAATACGCATCTCAACGCACT	Exon-4	1161
5	TGATAGGATGCAGGTATGCTGA	TTTCTGCTGCCAAGTGACTACA	Exon-5	1018
6	TAATATGCATCAGGACTAGTAGGT	AAATGATGAAGACTCAGGGAGC	Exon-6	1160
7	TCTGCTTTGAGACACCTTGA	TGAGCTGATGAATACTGCGT	Exon-7	994
8	GAACCCTGGAATCCTGATTAGA	CTTCAGGTAATGCGAAAGGACA	Exon-8	1052
9	GAGTTGTAGACATTCGCTGAGA	TGTGGGAGAGCTAGTCATTCAT	Exon-9	1582
10	GCAAAGTTCACTCAGATGTCAC	TTCTGGAGGAGATGAGACATTC	Exon-10, 11, 12, 13	1569
11	AGATCCACGGTCAAGTTGACAG	CACAGGAAAGAACCACGGTAAG	Exon-14	1117
12	GCCATCTAATTCCTAGGGATTG	TCTGTTAGCCTGGTTTCCTAAG	Exon-15, 16	1438
13	GAAACCAGGCTAACAGAATAGG	CGCATGCTGTCATCAGAAAGTA	Exon-17	1476
14	GGTGATCCACTTATTTTCAGAGG	CCAACTCTCATTCTCTACGGT	Exon-18, 19, 20	1647
15	CAAGTGCCTGGTTCTCAATGGA	GATGACATTCTGAAGCTGGTCG	Exon-21	1390

positions have been compared with NCBI database (Btau_4.2) and previously reported SNP positions using Bta_4.0.6 by Fontanesi et al. (2009).

RESULTS

The 18,378 bp KIT gene has been sequenced in this study, including all 21 exons (2934 bp) and partial intronic and flanking regions (5' and 3') in four different cattle breeds (black Hanwoo, stripe Hanwoo, yellow Hanwoo and Holstein). Based on the investigation of nineteen sequences, 126 polymorphisms, which include 23, 100 and 3 for exons, introns and 3'-untranslated region, respectively, were identified. Among these, two intronic indels, TTCTC and TCT in intron 3 and intron 5, respectively, were also identified. Further investigation of 23 exonic polymorphisms indicates that fifteen missense and eight silence mutations were observed (Fig. 1). By comparing the NCBI database SNP reference assembly, forty three SNPs were previously identified from our 126 polymorphisms. Based on the comparison of 111 polymorphic positions in the Fontanesi et al. (2009), forty seven polymorphisms were identical (Table 2). The neighbor-joining phylogenetic tree of the nine sequences from four different colored breeds has been estimated (Fig. 2). The white spotting cattle breed, Holstein, is indicated a clear breed-specific cluster in the phylogenetic tree. On the other hand, three different Korean cattle breeds were not separate well with the KIT polymorphisms. In case of black Hanwoo, three samples (BH1, BH3, BH5) were in the same cluster and other two samples (BH2, BH4) were clustered with stripe Hanwoo and yellow Hanwoo, respectively. Yellow Hanwoo samples were widely spread in the phylogenetic tree, indicating their genetic diversity within the breed. Finally, four samples of stripe Hanwoo were scattered

in four clusters, which clustered with black and yellow Hanwoo together. Base on the Fontanesi et al. (2009), twelve divergent haplotypes were discovered in Holstein cattle breed and coalescent replications did not indicate any significant deviation between the expected and observed number of haplotypes based on the number of segregating sites. In case of Hereford, coat color pattern was endorsed to the presence of the S^H allele at the spotting locus (Fontanesi et al., 2009). Heterogeneity may be affecting the measure of spotting as phenotype in Holstein because of a few QTL alleles were mapped near the KIT region (Reinsch et al., 1999).

DISCUSSION

The KIT gene is affecting coat color pattern in different modes of inheritance with the presence of multi allelic polymorphisms at the S locus (Olson, 1999). Also, constituted linkage mapping has been focused S locus near KIT gene on BTA6 (Grosz and MacNeil, 1999). Previously, identified polymorphisms showed the evidence of selection signatures at those loci in the Hereford breed (Fontanesi et al., 2009). In the Holstein and Simmental breeds, proportional white spotting coat color QTL was mapped on the same chromosome and this QTL was also confirmed in a Holstein $\times$ Jersey cross (Reinsch et al., 1999; Lui et al., 2009). Our results of the KIT gene, 126 polymorphisms were identified and forty seven of them were the same with the comparison of reported 111 polymorphisms by Fontanesi et al. (2009). Due to the small number of samples for the experiment, we did not consider the haplotype analysis. However, the identification of polymorphisms in the KIT gene from three different colored Korean cattle and Holstein breed, were performed.

The KIT gene sequence (GenBank accession number:

Fig. 1. Identified KIT gene polymorphisms in this study. The detailed information of the polymorphisms is appeared in Table 2.

Table 2. Identified KIT gene polymorphisms in four different cattle breeds

Sl no.	Gene region-no. of polymorphism	SNP position on Btau_4.2	Amino acid changes	Bta 4.0:6 position	NCBI db	Polymorphic allele genotype (breed) ¹⁾
1	Intron-1-1	g.72822409C>T		72741461	rs109230396	CT (YH)
2	Intron-1-2	g.72822413C>A		—	—	CA (HL)
3	Intron-1-3	g.72822641G>C		—	—	GC (SH)
4	Intron-1-4	g.72822729G>T		—	—	GT, TT (YH)
5	Intron-1-5	g.72860184C>T		—	—	TT (BH, SH, YH)
6	Intron-1-6	g.72860320G>T		—	—	GT (BH)
7	Intron-1-7	g.72860322G>A		—	—	AA (BH, HL), GA (YH)
8	Exon-2-1	g.72860724C>T	Thr>Thr	72779776	rs109344937	TT (BH, SH, YH)
9	Exon-3-1	g.72864030G>A	Glu>Lys	—	—	GA (HL)
10	Exon-3-2	g.72864033G>A	Glu>Lys	—	—	GA (HL)
11	Exon-3-3	g.72864093C>T	Thr>Met	—	—	CT (BH, SH), TT (SH)
12	Exon-3-4	g.72864098T>G	Cys>Gly	—	—	TG (HL)
13	Exon-3-5	g.72864130A>G	Thr>Thr	72783182	rs109314357	AG (BH, SH, YH), GG (YH)
14	Exon-3-6	g.72864165G>A	Arg>Lys	—	—	GA (SH, HL)
15	Exon-3-7	g.72864217G>A	Gln>Gln	—	—	GA (HL)
16	Exon-3-8	g.72864238G>A	Ser>Ser	—	—	GA (HL)
17	Intron-3-1	g.72864241G>A		—	—	GA (HL)
18	Intron-3-2	g.72864319G>A		—	—	GA (HL)
19	Intron-3-3	g.72864322>TTCTC		72783378	rs109704112	TTCTC (YH)
20	Intron-3-4	g.72864710G>A		72783762	rs110385675	AA (SH)
21	Intron-3-5	g.72864922A>C		—	—	CC (YH)
22	Intron-3-6	g.72865053A>G		—	—	GG (BH)
23	Intron-3-7	g.72865143A>C		72784195	rs110692488	CC (BH)
24	Intron-3-8	g.72865264T>G		72784316	rs110065637	GG (BH, SH, YH), GT (BH)
25	Intron-4-1	g.72865526A>C		—	—	AC (YH)
26	Intron-5-1	g.72869972A>G		72789024	rs110388507	AG (YH), GG (BH, YH)
27	Intron-5-2	g.72870140C>T		72789192	rs110307161	CT (BH, SH, YH), TT (BH, YH)
28	Intron-5-3	g.72870154A>G		72789206	rs110741671	AG (BH, SH, YH), GG (BH, SH, YH)
29	Intron-5-4	g.72873893A>G		—	—	AG (SH)
30	Intron-5-5	g.72873896T>G		—	—	TG (SH)
31	Intron-5-6	g.72873899C>G		—	—	CG (SH)
32	Intron-5-7	g.72873994A>G		—	—	AG (SH)
33	Intron-5-8	g.72874026G>A		—	—	GA (HL)
34	Intron-5-9	g.72874083G>A		72793135	rs110893852	GA (YH), AA (HL)
35	Intron-5-10	g.72874110A>G		—	—	AG (HL)
36	Intron-5-11	g.72874145A>G		—	—	AG (HL)
37	Intron-5-12	g.72874151T>G		—	—	TG (HL)
38	Intron-5-13	g.72874156T>G		—	—	TG (HL)
39	Intron-5-14	g.72874202G>A		—	—	GA (HL)
40	Intron-5-15	g.72874213T>A		—	—	TA (SH)
41	Intron-5-16	g.72874259G>T		—	—	GT (HL)
42	Intron-5-17	g.72874269G>A		—	—	GA (HL)
43	Intron-5-18	g.72874306C>T		72793358	rs109236495	CT (BH, SH, YH), TT (BH, YH)
44	Intron-5-19	g.72874322G>A		—	—	GA (HL)
45	Intron-6-1	g.72876489C>T		72795541	rs108989845	CC (BH, SH, YH)
46	Intron-6-2	g.72876534A>G		72795586	rs109078616	AA, GG (BH, SH, YH)
47	Intron-6-3	g.72876547T>G		72795599	—	TT, GG (BH, SH, YH)
48	Intron-7-1	g.72876955G>A		—	—	GA (SH)
49	Intron-7-2	g.72876966G>A		—	—	GA (BH)
50	Intron-7-3	g.72876979A>C		—	—	CC (SH, YH)
51	Intron-7-4	g.72876985G>A		—	—	AA, GG (BH, SH, YH)
52	Intron-7-5	g.72877054G>A		72796106	rs109343256	AA, GG (BH, SH, YH)
53	Intron-7-6	g.72892903A>G		—	—	AG (SH)
54	Intron-7-7	g.72892911C>G		—	—	CG (SH)
55	Intron-7-8	g.72892958T>G		—	—	TG (SH)
56	Intron-7-9	g.72892973A>G		—	—	AG (SH)
57	Intron-7-10	g.72892977T>C		72812030	rs110595646	CC (SH)
58	Intron-7-11	g.72893028T>G		—	—	TG (SH)
59	Intron-7-12	g.72893033C>T		72812085	—	TT (BH), CT (YH)
60	Intron-7-13	g.72893036C>A		72812088	rs110602058	AA (SH)
61	Intron-7-14	g.72893124G>A		72812176	—	GA (SH)
62	Intron-8-1	g.72894182A>C		72813234	rs110825736	CC (BH, YH), AC (SH)

63	Intron-8-2	g 72894310G>A	72813362	rs110917062	GA (SH)
64	Intron-8-3	g 72894499C>A	72813551	-	CA (HL)
65	Intron-8-4	g 72894642G>A	72813694	rs110871960	AA (BH), GA (SH, YH)
66	Intron-8-5	g 72894738T>G	-	-	TG (BH, SH, YH)
67	Intron-8-6	g 72894769C>G	72813821	rs109921120	CG (SH)
68	Intron-8-7	g 72894887G>C	-	-	GC (BH), CC (YH)
69	Intron-9-1	g 72895482C>T	-	-	CT (SH)
70	Intron-9-2	g 72895494C>T	-	-	TT (BH)
71	Intron-11-1	g 72897088T>C	-	-	CC (BH, YH)
72	Exon-13-1	g 72897140C>A	-	-	AA (BH)
73	Intron-13-1	g 72897473C>A	72816525	rs110118210	AA (BH)
74	Intron-13-2	g 72898004A>G	72817056	rs109278474	GG (BH, YH)
75	Intron-13-3	g 72898035G>A	-	-	GA (HL)
76	Intron-13-4	g 72898053C>T	-	-	TT (HL)
77	Intron-13-5	g 72898104T>C	72817156	rs110813009	CC (BH), TC (YH)
78	Intron-13-6	g 72898123A>G	-	-	AG (SH)
79	Intron-13-7	g 72898154T>G	-	-	TG (BH, SH, YH, HL)
80	Intron-13-8	g 72898315T>C	72817367	rs110272569	CC (BH, YH), TC (SH)
81	Intron-13-9	g 72898357G>A	-	-	GA (HL)
82	Intron-13-10	g 72898403G>A	72817455	rs110550547	GA (SH)
83	Intron-13-11	g 72898563T>C	72817615	rs109750754	CC (BH, SH, YH)
84	Intron-14-1	g 72898930T>A	72817982	rs110171094	AA (BH, YH)
85	Intron-14-2	g 72900606T>C	72819658	rs109891739	CC (BH, YH), TC (SH)
86	Exon-15-1	g 72900672A>T	-	-	AT (SH)
87	Intron-15-1	g 72900744T>G	-	-	TG (SH)
88	Intron-15-2	g 72900839>TCT	72819891	rs111028244	TCT (BH)
89	Intron-15-3	g 72901014G>C	-	-	CC (BH, SH, YH)
90	Intron-15-4	g 72901114A>G	72820166	rs109431569	AG (BH, HL), GG (SH, YH)
91	Intron-15-5	g 72901140G>T	72820192	rs109203908	TT (BH)
92	Intron-15-6	g 72901156T>C	72820208	rs109948049	CC (SH)
93	Exon-16-1	g 72901307G>A	72820359	rs110818069	AA (BH), AG (YH)
94	Intron-16-1	g 72901423T>C	-	-	CC (BH, YH)
95	Intron-16-2	g 72901449T>C	72820501	rs110484938	CC (BH, SH, YH)
96	Intron-16-3	g 72902057G>T	72821109	rs109867065	TT (BH), GT (YH)
97	Intron-16-4	g 72902108T>G	-	-	TG (BH, YH, HL)
98	Intron-16-5	g 72902122A>G	72821174	rs110601510	AG (BH, YH, HL), GG (YH, HL)
99	Intron-16-6	g 72902166A>C	-	-	AC (BH, SH, YH)
100	Intron-16-7	g 72902207G>A	-	-	GA (BH, SH, YH)
101	Intron-16-8	g 72902221T>G	-	-	TG (SH)
102	Intron-16-9	g 72902326T>C	-	-	TC (SH, HL)
103	Exon-17-1	g 72902377T>C	-	-	TC (SH)
104	Exon-17-2	g 72902390T>C	-	-	TC (BH, SH, YH), CC ((SH, YH)
105	Exon-17-3	g 72902434A>G	-	-	AG (BH, YH)
106	Exon-17-4	g 72902455T>G	-	-	TG (SH)
107	Intron-17-1	g 72902967C>T	72822019	rs109283899	TT (BH)
108	Intron-17-2	g 72905210A>G	-	-	AG (HL)
109	Intron-17-3	g 72905348A>G	-	-	AG (HL)
110	Exon-18-1	g 72905401G>A	-	-	GA (SH, HL)
111	Exon-18-2	g 72905403G>A	-	-	GA (HL)
112	Exon-18-3	g 72905464T>G	-	-	TG (SH, YH)
113	Exon-19-1	g 72905642G>A	-	-	GA (HL)
114	Intron-20-1	g 72906318T>C	72825370	rs109479879	CC (BH), TC (SH)
115	Intron-20-2	g 72906350A>G	72825402	rs109646720	AG (BH, SH, HL), GG (BH, SH, YH)
116	Intron-20-3	g 72906391T>A	72825443	rs110030468	AA (BH), TA (SH)
117	Intron-20-4	g 72906422T>C	72825474	rs110697952	CC (BH), TC (SH)
118	Intron-20-5	g 72906517A>C	-	-	AC (SH, YH)
119	Intron-20-6	g 72907135G>A	-	-	GA (HL)
120	Intron-20-7	g 72907240G>A	-	-	GA (HL)
121	Exon-21-1	g 72907279G>A	-	-	GA (HL)
122	Exon-21-2	g 72907359G>A	-	-	GA (HL)
123	Exon-21-3	g 72907361G>A	-	-	GA (HL)
124	3' UTR-1	g 72907371G>A	-	-	GA (YH)
125	3' UTR-2	g 72907444T>C	-	-	TC (YH)
126	3' UTR-3	g 72907484T>C	72826536	rs110146056	CC (SH)

¹⁾ BH = Black Hanwoo, SH = Stripe Hanwoo, YH = Yellow Hanwoo, HL = Holstein.

Fig. 2. Construction of neighbor-joining (NJ) phylogenetic tree using Kimura 2-parameter model from sequences of individual animals with 1000 bootstrap replications (A) and using SNPs for identify the phylogenetic relationship among populations (B) in Holstein and three different colored Hanwoo breeds.

NC_007304) is included in the Btau_4.6.1 version and SNP data base was used the previous version of Btau_4.2. Investigation of two polymorphisms (g.72779776C>T and g.72783182A>G on position Bta_4.0:6) were confirmed as complex heterogeneity of the KIT gene, having different coat color patterns, which determine the spotting phenotype in Holstein-Friesian and Simmental breeds (Fontanesi et al., 2010). Based on the QTL studies, multi-allelic polymorphisms of spotting have been mapped on BTA6 in the region covering the KIT gene (Reinsch et al., 1999; Liu et al., 2009). Our investigation also included these two polymorphisms. Analysis of heterogeneity in the bovine KIT gene was also observed for the SNP in intron 3 (g.72784316G>T in Bta_4.0:6), which were not fixed in spotted and non-spotted Icelandic and Nordic cattle breeds (Olsen et al., 2000; Klungland et

al., 2000).

Recently, evidence of selection signatures in the bovine genome has been reported for the different breeds, where no clear evidence of selective sweep in the region of BTA6 including the KIT gene (Hayes et al., 2009; MacEachern et al., 2009; The bovine HapMap Consortium, 2009). This might be due to the lack of information of the identification polymorphisms for this analysis. In our results, we discovered new polymorphisms which compared with the SNPs in NCBI database (Btau_4.2) and previously identified polymorphisms (Bta_4.0:6) in the KIT gene (Fontanesi et al., 2009). Based on this experiment, the causative KIT polymorphisms affecting the different colored Hanwoo breeds were not identified. However, this result provides valuable information of KIT gene polymorphisms, which were not identified previously, and may use as further analysis for affecting coat color patterns in cattle.

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