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<td>Ichii, Osamu; Kamikawa, Akihiro; Otsuka, Saori; Hashimoto, Yoshiharu; Sasaki, Nobuya; Endoh, Daiji; Kon, Yasuhiro</td>
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Title: Overexpression of interferon-activated gene 202 (Ifi202) correlates with the progression of autoimmune glomerulonephritis associated with the MRL chromosome 1

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Running head: Ifi202 in murine autoimmune glomerulonephritis
Summary

B6.MRLc1(82-100) congenic mice carrying the telomeric region of lupus-prone MRL chromosome 1 develop autoimmune glomerulonephritis (GN). The GN susceptibility locus of B6.MRLc1(82-100) contains the Interferon activated gene 200 (Ifi200) family, which consists of Ifi202, 203, 204, and 205. Recently, Ifi202 was suggested as a candidate gene for murine lupus. In this study, we assessed the association between Ifi200 family and GN in several disease models. We compared the expression of Ifi200 family members in 24 organs between the C57BL/6 and B6.MRLc1(82-100). The expressions of Ifi200 family members differed between strains, especially, the most dramatic differences appeared in Ifi202 expression. Briefly, in the blood, immune organs, lungs, and testes was higher in B6.MRLc1(82-100) mice. In the kidney and immune organs, only Ifi202 expression increased with the development of GN in B6.MRLc1(82-100), and significant differences from the C57BL/6 were observed even before disease onset. Ifi202 expression in the kidneys of BXSB, NZB/WF1, and MRL/lpr was also significantly high in the early- and late-disease stages. Furthermore, laser microdissection-RT-PCR analysis confirmed the high Ifi202 expression in all areas of B6.MRLc1(82-100) kidneys. In conclusion, among Ifi200 family, Ifi202 expressions in the kidney and immune organs significantly increased with GN progression.
**Introduction**

Interferons (IFNs), including types I (IFN-α and IFN-β) and II (IFN-γ), are a family of cytokines, which play an important role in antiviral and natural immunity. Binding of an IFN to its receptor induces the transcriptional activation of IFN-stimulated genes via the activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. The IFN activated gene 200 (Ifi200) family, including Ifi202, Ifi203, Ifi204, and Ifi205, consists of genes induced by IFNs and are localized on the murine telomeric region of Chromosome 1 (Chr.1) (95 cM). These genes encode highly homologous proteins and are suggested to play an important role in the regulation of cellular growth, differentiation, survival, and death *in vitro*. In humans, IFI16, MNDA, AIM2, and IFIX, which have high homology with the murine Ifi200 family, are also localized on Chr.1q21–23.

Glomerulonephritis (GN) is caused by certain infections, tumors, drugs, and autoimmune diseases. Systemic lupus erythematosus (SLE) is a polygenic autoimmune disease characterized by multiorgan inflammation and the production of autoantibodies. These autoantibodies form immune complexes and cause autoimmune GN called lupus nephritis, which is a major cause of morbidity and mortality both in humans with SLE and animal models of lupus. Some reports suggest that SLE patients have increased serum IFN-α levels and their peripheral blood cells show increased IFN-α expression, which indicate the activation of IFN-α signaling. In addition, it has been suggested that increased IFN-inducible gene expression is associated with the pathophysiology of lupus nephritic patients.

BXSB, NZB/WF1, and MRL mice are important animal models of autoimmune diseases. Bxs3 (71-99 cM) and Nba2 (92-94 cM) in the telomeric region of murine
Chr.1 were reported as lupus susceptibility loci in BXSB and NZB-derived genomes, respectively.\textsuperscript{9} Recently, \textit{Ifi202} in the \textit{Bxs3} and \textit{Nba2} loci was identified as a major susceptibility gene by microarray analysis.\textsuperscript{10, 11} Furthermore, we discovered the GN-susceptibility locus, named \textit{MRL autoimmune glomerulonephritis (Mag)}, which is localized in the telomeric region of MRL Chr.1, by generating B6.MRLc1(82-100) congenic mice having the telomeric region of MRL-type Chr.1 (82-100 cM) and the C57BL/6 background.\textsuperscript{12} Aged B6.MRLc1(82-100) mice, especially females, exhibit glomerular proliferative and membranous lesions with splenomegaly and serum autoantibody levels.\textsuperscript{13, 14}

The \textit{Ifi200} family is located in the \textit{Mag} locus.\textsuperscript{12-14} In the present study, we determined whether the expression of \textit{Ifi200} family members changed with the development of GN in the B6.MRLc1(82-100) strain as well as several other murine lupus models. We found that of all the \textit{Ifi200} family members, the overexpression of only \textit{Ifi202} correlated with the onset of GN in the \textit{Ifi200} family.
Materials and Methods

Animal maintenance and sample preparation

We adhered to the guidelines reported in the “Guide for the Care and Use of Animals” of the Graduate School of Veterinary Medicine, Hokkaido University. The B6.MRLc1(82-100) mouse model for GN was generated by us previously. Female C57BL/6 mice (control), and male BXSB, female NZB/WF1, and female MRL/lpr mice (models of lupus nephritis) were purchased from an animal-breeding company (Japan SLC). All animals were maintained in specific pathogen-free (SPF) conditions. B6.MRLc1(82-100) mice of both sexes aged 1, 4, and 12 months were used in this study. C57BL/6 mice and mice of the lupus models were divided into early- and late-stage groups according to the severity of the disease: early stage (4 months) and late stage (5 months for BXSB, 7 months for NZB/WF1 and MRL/lpr, and 6 months for C57BL/6). Animals were sacrificed under deep anesthesia (induced by 50 mg/kg pentobarbital sodium administered intraperitoneally) by exsanguination from the carotid arteries, and organ samples were collected. The organs were fixed with 4% paraformaldehyde (PFA) for histological analysis. The remaining fresh tissue samples were stored in RNAlater solution (Ambion) for analysis of mRNA expression or frozen in liquid nitrogen after embedding in OCT solution (Tissue-tek; Sakura Finetek) and stored at –80°C for laser microdissection (LMD).

Histopathological analysis

Paraffin-embedded tissue sections (2 µm) of PFA-fixed kidneys were stained with Masson’s trichrome (MT) or periodic acid-Schiff (PAS) and observed under a light
microscope. To assess the severity of glomerular damage, 100 glomeruli per kidney were scored for the degree of PAS-positive deposition, cell proliferation, membrane hypertrophy, and podocyte adhesion to the parietal layer of the renal corpuscle (Ichii et al. 2008a). Briefly, glomeruli were scored according to the following criteria: grade 0, no recognizable lesion in glomeruli; grade 1, a little PAS-positive deposition, mild cell proliferation, mild membranous hypertrophy, and/or partial podocyte adhesion to the parietal layer of the renal corpuscle; grade 2, segmental or global PAS-positive deposition, cell proliferation, membranous hypertrophy, and/or glomerular hypertrophy; grade 3, the same as grade 2 with PAS-positive deposition in 50% of regions of glomeruli and/or severe podocyte adhesion to the parietal layer of the renal corpuscle; grade 4, disappearance of capillary and capsular lumina, global deposition of PAS-positive material, and/or periglomerular infiltration of inflammatory cells and fibrosis.

Reverse transcriptase-polymerase chain reaction

To analyze the mRNA expression of Ifi200 family genes and Actb, total RNA was isolated from 24 organs by using the Trizol reagent (Invitrogen). The purified total RNA was treated with DNase (Nippon Gene) for DNA digestion and reverse-transcribed to cDNA by using ReverTra Ace (Toyobo) and oligo dT primers (Invitrogen). Polymerase chain reaction (PCR), for the amplification of cDNA, was performed with ExTaq (Takara) under the following conditions: 5 min at 95°C; 35 cycles each of 40 s at 95°C, 30 s at 58°C, and 30 s at 72°C; and 5 min at 72°C. Details of the specific primers used for each gene are shown in Table 1.

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Quantitative real-time PCR

Quantitative real-time PCR (QPCR) analysis was performed using the Brilliant SYBR Green QPCR Master Mix (Stratagene) and the real-time thermal cycler (MX 3000; Stratagene). The specific primers for each gene were the same as those used for reverse transcriptase- (RT)-PCR (Table 1). The amplification conditions were as follows: 10 min at 95°C; 40 cycles each of 10 s at 95°C, 20 s at 58°C, and 20 s at 72°C; and 1 cycle of 10 s at 95°C, 20 s at 58°C, and 20 s at 95°C. The ROX dye was included in each reaction to normalize the non-PCR-related fluctuations in fluorescence. The amplification specificity of all the PCR reactions was confirmed by melting curve analysis. Non-template controls were included for each primer pair to assess any significant levels of contaminants. The expression data of Ifi200 family genes were normalized to the expression of Actb.

LMD and RT-PCR

LMD was performed as previously reported. First, 5 μm-thick cryosections obtained from fresh kidneys were mounted on glass slides precoated with LMD films (Meiwafosis) and fixed with absolute alcohol containing 5% acetic acid for 3 min at 4°C. After staining with 1% toluidine blue for 10 sec, LMD was performed on the glomeruli (GL): cortex (CO), except the glomeruli: outer medulla (OM): inner medulla (IM): and perivascular lesion (PVL) by using Ls-Pro300 (Meiwafosis), according to the manufacturer’s protocol. All procedures were performed in RNase-free conditions.

Total RNA purified with RNAqueous (Ambion) and Turbo DNase (Ambion) was reverse-transcribed to cDNA by using the reaction mixture containing random
primers (Promega) and ReverTraAce (Toyobo) for 1 h at 42°C. cDNA was adjusted to a concentration of 0.25 µg/µl and used for the PCR with Ex Taq and appropriate primer pairs (Table 1). The amplified samples were electrophoresed on a 2% agarose gel containing ethidium bromide, and photographed under an ultraviolet lamp.

**Statistical analysis**

Results were expressed as median ± interquartile range and statistically analyzed using the nonparametric Mann-Whitney U test ($p < 0.05$).
Results

Chromosome 1 and GN of B6.MRLc1(82-100) mice

Chr.1 of B6.MRLc1(82-100) mice and the names and the relative distances of the genes located on this chromosome are depicted on the basis of data obtained from the Entrez cross-database (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi) (Figure 1). The MRL-type congenic interval included the Ifi200 family locus and other immune- or cell proliferation-associated genes such as Fas ligand (Fasl), selectin (Sel), Fc gamma receptor (Fcgr), and exonuclease1 (Exo1). In the B6.MRLc1(82-100) strain, glomerular damage, including mild cell proliferation and membrane hypertrophy with immune-complex deposition, was observed from approximately 6 months of age and worsened thereafter. Moreover, disease was more severe in female mice than in male mice (Figure 1).12-14

mRNA expression of Ifi200 family genes in various organs of adult mice

At 4 months of age, the mRNA expression of Ifi200 family genes was qualitatively analyzed by RT-PCR in 24 organs of C57BL/6 and B6.MRLc1(82-100) mice (Figure 2). The cDNA concentration of each sample was adjusted to 12.5ng/µl. In general, Ifi202 expression in these organs was higher in the B6.MRLc1(82-100) mice than in the C57BL/6 mice. In particular, the mRNA expression in the blood, lymph nodes, spleen, lungs, and testes of the B6.MRLc1(82-100) mice was higher than that in the C57BL/6 mice. In contrast, no marked differences in the expression of Ifi203 were observed between the 2 strains. The strain differences of Ifi204 and Ifi205 mRNA expressions differed among organs. Briefly, the expression of Ifi204 mRNA in the skeletal muscle, lymph nodes, stomach, and testes was higher in the
C57BL/6 strain; conversely, its expression in the lungs and kidneys was higher in the B6.MRLc1(82-100) strain. *Ifi205* mRNA expression in the eyes was higher in the B6.MRLc1(82-100) mice and that in the skeletal muscle and intestine was higher in the C57BL/6 mice.

**mRNA expression of *Ifi200* family genes in the kidneys and immune organs of young mice**

At 1 month of age, marked differences in the expression of *Ifi202* in the kidneys, spleen, and thymus were observed between the two strains; its expression in the B6.MRLc1(82-100) strain was higher than that in the C57BL/6 strain (Figure 3). These results were also confirmed by QPCR analysis (Figure 4a–c). The B6.MRLc1(82-100) mice showed significantly higher *Ifi202* mRNA expression in these organs than the C57BL/6 mice.

**Glomerulonephritis and mRNA expression of *Ifi202***

The time-course of *Ifi202* mRNA expression in the kidneys of B6.MRLc1(82-100) mice, as determined using QPCR, was compared with the histoplanimetrical GN scores evaluated from PAS-stained sections (Figure 5a–e). *Ifi202* mRNA expression in B6.MRLc1(82-100) kidneys increased with increase in the GN scores. At 6 and 12 months, *Ifi202* mRNA expression in the B6.MRLc1(82-100) mice was significantly higher than that in the C57BL/6 mice (Figure 5f).

**mRNA expression of *Ifi202* in the kidneys of other GN models**

In the quantitative PCR analysis for *Ifi200* family in several lupus models,
Ifi202, Ifi203, and Ifi204 mRNA expressions were significantly different from those of C57BL/6 (Figure 6a-c). In Ifi203 mRNA expressions of the early stage, BXSB and MRL/lpr showed significantly higher values than those of C57BL/6 (Figure 6b). In the late stage, MRL/lpr showed significantly higher Ifi203 mRNA expressions than those of C57BL/6 (Figure 6b). In Ifi204 mRNA expressions, NZB/WF1 showed significantly lower values than those of C57BL/6 in the early stage (Figure 6c). Importantly, the most dramatic change was observed in Ifi202 expressions (Figure 6a). In the early stage of the disease, lupus mouse models including BXSB, NZB/WF1, and MRL/lpr showed significantly higher Ifi202 mRNA expression in the kidney than that showed by the C57BL/6 model (Figure 6a, see Y-axis). The expression further increased in the late stage of the disease (Figure 6a).

Local expression of Ifi202 mRNA in the kidneys of a GN model

The spatial expression of Ifi202 mRNA in the kidneys of 12-month-old B6.MRLc1(82-100) mice was assessed by LMD targeting the GL, CO, OM, IM, and PVL (Figure 7a and 7b). After LMD, RT-PCR was performed to analyze the expression of Ifi202 and component-specific genes such as Wt1 (glomerulus), Aqp1 (proximal tubules), Slc12a1 (distal tubules), and Aqp2 (collecting ducts) in (Figure 6c). The success of LMD was confirmed by the mRNA expression of Wt1 (GL, PVL), Aqp1 (CO, OM), Slc12a1 (OM), and Aqp2 (OM, IM); further, strong Ifi202 expression was observed in all these areas.
Discussion

B6.MRLc1(82-100) congenic mice develop autoimmune GN with splenomegaly and increase in serum autoantibody levels. The susceptibility locus for this disease (Mag locus) contains the MRL-type Ifi200 gene family. Among the Ifi200 family genes, Ifi202 was dramatically overexpressed in the immune organs and the kidneys of B6.MRLc1(82-100) mice. Furthermore, this observation was also confirmed by analyzing the kidneys of other GN models such as BXSB, NZB/WF1, and MRL/lpr. Similarly, in previous studies, B6.Nba2 mice and Balb/c.c1(77-105) mice, which possess the lupus-prone NZB Chr.1, showed increased Ifi202 expression. In addition, microarray analysis has revealed that Ifi202 is overexpressed in B10.Yaa.Bxs2/3 mice, which possess the telomeric region of BXSB Chr.1; thus, this gene was suggested to be a candidate gene for lupus susceptibility. Our results suggest that the GN-susceptibility locus derived from MRL Chr.1 Mag is responsible for Ifi202 overexpression in the B6.MRLc1(82-100).

Interestingly, compared with the C57BL/6 mice, B6.MRLc1(82-100) mice tended to have higher Ifi202 expression in not only the immune organs and kidneys but also other organs. Therefore, we considered that Ifi202 overexpression in B6.MRLc1(82-100) mice may not be caused by the immunological secondary effects but differences in the mouse genome. Some genetic mechanisms of Ifi202 overexpression, especially polymorphisms in the Ifi202 promoter region among various mouse strains, have been suggested in several studies. At least 10 polymorphic sites have been identified in the 5′-regulatory region of the Ifi202 gene between the C57BL/6 and NZB genomes, and one NZB-specific polymorphism was predicted to result in a TATA sequence that binds to transcription factors.
Furthermore, the C57BL/6 and C57BL/10 strains show lower \textit{Ifi202} expression and have a T allele downstream of the IFN-stimulated response element-like sequence, whereas the lupus-prone MRL and NZB strains have a C allele.\textsuperscript{11} On the basis of these findings, we considered that \textit{Ifi202} overexpression in B6.MRLc1(82-100) may be caused by promoter-region polymorphisms derived from the MRL genome.

In the present study, \textit{Ifi202} overexpression in B6.MRLc1(82-100) was clearly detected even before disease onset. Furthermore, in the kidney, the expression of \textit{Ifi202} markedly increased with the development of GN but did not show any region-specific pattern. \textit{IFI16}, the human homolog of mouse \textit{Ifi202}, was found to be mainly expressed in endothelial cells, whereas \textit{Ifi202} was found to be expressed in various cells such as bone marrow cells, fibroblasts, lymphocytes, and myoblasts \textit{in vitro}.\textsuperscript{4, 17-20} On the basis of these findings, it was proposed that the overexpression of \textit{Ifi202} may occur in not only glomerular and tubular cells but also interstitial cells including lymphocytes in the kidneys of B6.MRLc1(82-100) mice. \textit{Ifi202} protein is generally induced by IFNs and has been suggested to contribute to the regulation of cellular differentiation, proliferation, and survival by controlling the activities of several transcription factors.\textsuperscript{17-20} Therefore, because of the presence of \textit{Ifi202}-overexpressing cells in the kidney and other organs, we speculated that the autoimmune disease models, including B6.MRLc1(82-100), have some characteristic background similarities, which elicit a strong response to immunological stimulations such as those cause by IFNs.

Recently, \textit{Ifi202} was suggested to be a lupus-susceptibility gene in the Bxs3 and Nba2 loci derived from BXSB and NZB genomes, respectively.\textsuperscript{10, 11} Although the Nba2 locus derived from the NZB-type Chr.1 (79–109 cM) and including \textit{Ifi202} is
associated with splenomegaly and high serum levels of immunoglobulin (Ig)G antinuclear antibodies, it is insufficient to cause GN. Serum amyloid P-component (Apcs; Chr.1, 94 cM)-disrupted mice develop SLE-like severe GN and exhibit elevated serum autoantibody levels. However, it has been argued that the deficiency of Apcs alone does not cause these diseases and interactions between Apcs and other genes, especially Ifi202 is necessary. Furthermore, it has been suggested that the imbalance between the active and inhibitory Fc gamma receptor genes (Fcgrs; Chr.1, 92 cM) that are located in the Mag and are locally expressed in the glomeruli and dendritic cells greatly impact the pathogenesis of GN in the B6.MRLc1(82-100) strain. Future research should focus on determining whether Ifi202 overexpression alone can result in GN or whether its interaction with other genes on the congenic interval is required for disease onset.

In conclusion, we clarified that among the Ifi200 family genes, only Ifi202 was overexpressed in B6.MRLc1(82-100) mice and other murine models of lupus, such as BXSB, NZB/WF1, and MRL/lpr. This overexpression increased with the age-dependent progression of GN in B6.MRLc1(82-100). These findings suggest that Ifi202 may be a lupus-susceptibility genes located in the Mag.

Acknowledgements

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Figure legends

Figure 1 Schematic representation of B6.MRLc1(82-100) chromosome 1 and Ifi200 gene family

The MRL/MpJ type congenic interval is indicated by the bold line. Certain immune- or cell proliferation-associated genes located in the congenic interval, such as Fas ligand (Fasl), selectin endothelial (Sele), selectin lymphocyte (Sell), selectin platelet (Selp), Fc gamma receptor (Fcg), interferon-activated gene (Ifi), and exonuclease1 (Exo1), are indicated on the right. The Ifi200 gene family, which is composed of Ifi202, Ifi203, Ifi204, and Ifi205, is localized at 95 cM. Aged B6.MRLc1(82-100) mice showed severe glomerulonephritis. Further, the immune complex-depositions and membranous and proliferative lesions associated with glomerulonephritis were more severe in the female mice than in the male mice (panels).

Figure 2 mRNA expression of Ifi200 family members in various organs of adult mice

RT-PCR analysis showing the expression profiles of Ifi200 family members in 24 organs of 4-month old female C57BL/6 (upper lane) and B6.MRLc1(82-100) (lower lane) mice. The cDNA of testes was obtained from male mice at the same age. The cDNA concentration of each sample was adjusted to 12.5ng/µl. Size markers are represented on the left.

Figure 3 mRNA expression of Ifi200 family members in the kidney and immune organs of young mice

RT-PCR analysis of the expression profiles of Ifi200 family members in the
kidneys, spleen, and thymus of 1-month-old female C57BL/6 (upper lane) and B6.MRLc1(82-100) (lower lane) mice. Size markers are represented on the left.

**Figure 4 Comparison of relative mRNA expression of *Ifi200* family members in the kidney and immune organs of mice.**

Quantitative real-time PCR analysis showing relative mRNA expression of *Ifi200* family members in the kidney (a), spleen (b), and thymus (c) of 1-month-old C57BL/6 and B6.MRLc1(82-100) mice. The expression of each *Ifi200* family member is normalized to that of *Actb* and is represented as the median ± interquartile range. *: Significant difference from the expression in C57BL/6 mice for the same gene (Mann-Whitney *U* test, *p* < 0.05). *n* = 4.

**Figure 5 Time course of glomerulonephritis scores and renal *Ifi202* expression**

Grade of Glomerulonephritis severity in B6.MRLc1(82-100) mice. (a) 0 grade, no recognizable lesion in glomeruli. (b) +1 grade, a little PAS-positive deposition, mild cell proliferation, mild membranous hypertrophy, and/or partial podocyte adhesion to the parietal layer of the renal corpuscle. (c) +2 grade, segmental or global PAS-positive deposition, cell proliferation, membranous hypertrophy, and/or glomerular hypertrophy (d) +3 grade, the same as grade 2 with PAS-positive deposition in 50% of regions of glomeruli and/or severe podocyte adhesion to the parietal layer of the renal corpuscle. (e) +4 grade, disappearance of capillary and capsular lumina, global deposition of PAS-positive material, and/or periglomerular infiltration of inflammatory cells and fibrosis. Scale bars, 20µm. The time-course of renal *Ifi202* mRNA expression in B6.MRLc1(82-100) and C57BL/6 mice, and the
histoplanimetrical glomerulonephritis scores of B6.MRLc1(82-100) mice (Φ). Each value of Ifi202 expression is normalized to that of Actb and both mRNA expression and histoplanimetrical scores are represented as the median ± interquartile range. *: Significant difference from Ifi202 expression in C57BL/6 mice at the same age (Mann-Whitney U test, p < 0.05). n = 3.

**Figure 6 Relative expression of Ifi202 mRNA in the kidneys of various lupus models**

Quantitative real-time PCR analysis showing Ifi202 expression in the kidneys of C57BL/6 mice and lupus models. Early disease stage was analyzed at 4 months. Late disease stage was analyzed at 5 months (BXSB), 6 months (C57BL/6), and 7 months (NZB/W F1 and MRL/lpr). Each value of Ifi202 mRNA expression is normalized to that of Actb, expressed as fold increases compared to C57BL/6 mice of early stage and represented as the median ± interquartile range. *: Significantly different from C57BL/6 at the same disease stage (Mann-Whitney U test, p < 0.05). n = 3.

**Figure 7 Local expression of Ifi202 mRNA in the kidneys of 12-month-old B6.MRLc1(82-100) mice**

RT-PCR analysis following laser microdissection targeting the glomeruli: cortex, except glomeruli; outer medulla; inner medulla; and perivascular lesion. Laser microdissection of the glomerulus (panels). The success of LMD was confirmed by analyzing the renal component-specific mRNA expression of genes such as Wt1 (glomerulus), Aqp1 (proximal tubules), Slc12a1 (distal tubules), and Aqp2 (collecting ducts). Size markers are represented on the left.
References


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* LMD: Laser-microdissection
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