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Original Article

Aging-related gene signature regulated by Nlrp3 predicts glioma progression

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Abstract: Aging is the strongest risk factor for glioma development, suggesting that molecular crosstalks between aging and tumorigenesis exist in many cellular pathways. Recently, Nlrp3 inflammasome have been shown to modulate several major cellular pathways such as inflammation and cell death and have been demonstrated to be an upstream target that controlled the process of brain aging. We proposed Nlrp3 inflammasome may serve as a possible molecular link between aging and glioma progression. In this study, we generated a aging-related gene signature that regulated by Nlrp3 in mouse hippocampus and demonstrated that this gene signature can distinguish subsets of glioma samples and predicts clinical outcome in radiotherapy-treated patients. In addition, using U87 and GL261 xenograft mouse glioblastoma model, we found that Nlrp3 inflammasome contributed to radiotherapy resistance in glioma. Ionizing radiation can induce Nlrp3 inflammasome expression; Nlrp3 inhibition reduced tumor growth and prolonged the survival of mouse following IR treatment; Nlrp3 inhibition reduced number of senescent cells induced by IR. These results above suggest that Nlrp3 inflammasome is an important molecular link between brain aging and glioma progression; the Nlrp3 gene signature may serve as a predictive biomarker for glioma patients.

Keywords: Nlrp3, radiotherapy, aging, gene signature, glioma

Introduction

Malignant gliomas are the most common and deadly primary brain tumors in adults. Although there is much progress in surgery, radiotherapy and chemotherapy for management of this disease, the prognosis is still poor [1]. Age is among the most robust single predictive factors in glioma incidence and shortened patient survival, increased age is strongly associated with increased cancer incidence and patient survival for all grades and types of glioma [2-4]. Despite the strong associations between patient age and glioma outcomes, the molecular link between aging and glioma progression is not fully understood.

The cytosolic nod-like receptor (NLR) pathway of innate immunity has gained a lot of attention in the tumor field due to its known involvement in promoting inflammation and immunity [5-7]. Several NLRs have so far been identified in humans i.e., NLRP1, NLRP3, NLRP6, NLRC4, and the HIN200 protein AIM2. NLRs can respond to both pathogen- and danger-associated molecular patterns, which have been shown to behave as tumor promoters via the induction of chronic inflammation, and then they initiate the inflammasome pathway, a cytosolic signaling apparatus that canonically activates caspase-1, and IL-1β and IL-18 thereafter. The activation of inflammasome plays diverse and sometimes contrasting roles in cancer development and therapy depending on the specific context [8-10].

Recent studies reported that Nlrp3 inflammasome was an upstream target that controlled the process of aging in brain [11]. Ablation of Nlrp3 inflammasome activity can reduce age-related inflammation in hippocampus, protect mice from age-related decline in cognition and

memory, and extend health span. As age is strongly associated with increased glioma incidence, malignancy and therapy resistance, we proposed that Nlrp3 inflammasome may serve as a possible molecular link between aging and glioma tumorigenesis and progression.

In this study, we generated a aging-related gene signature that regulated by Nlrp3 in mouse hippocampus and demonstrated that this gene signature can distinguish subsets of glioma samples and predicts clinical outcome in GSE 16011 and TCGA cohort. Previous studies reported that IR could induce premature senescence in glioma cells [12, 13]. As cell senescence has an intimate link with aging, we then examined whether Nlrp3 inflammasome is required for radiotherapy resistance in glioma. Using U87 and GL261 xenograft mouse glioblastoma model, we found that Ionizing radiation (IR) can induce Nlrp3 inflammasome expression; Nlrp3 inhibition reduced tumor growth and prolonged the survival of mouse following IR treatment. In addition, the results showed that Nlrp3 inhibition reduced number of senescent cells induced by IR.

To our knowledge, this is the first report that a aging-related gene signature regulated by a key molecule is effective in predicting the clinical outcome in glioma patients. Most importantly, we demonstrated for the first time that Nlrp3 inflammasome is an important molecular link between brain aging and glioma progression and radiotherapy resistance.

Materials and methods

Microarray data sources

Gene expression data GSE16011 and corresponding clinical data used in this study were obtained from GEO databases and related article [14]. For the TCGA cohort, GBM samples from 150 long-term survivors and 150 Short-term survivors were included in the study [15].

Generation of the aging-related gene signature regulated by Nlrp3 in hippocampus

Microarray data of three rat and one rhesus hippocampal aging studies were analyzed with an overlap analysis to generate the aging-related gene signature in hippocampus [16-19]. The genes that were regulated by Nlrp3 in hippocampal during brain aging has been derived by analyzing microarray data of GSE43034 [11]. At last, aging-related genes regulated by Nlrp3 were chose for the signature. NCBI Gene and NCBI HomoloGene databases were used to translate animal array probe sets to human homolog gene symbols.

Cell culture and shRNA

Human glioblastoma cell line U87 was obtained from Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS, Shanghai, China). Mouse glioblastoma cell line GL261 was obtained from American Type Culture Collection (ATCC). U87 and GL261 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone), 1% penicillin-streptomycin (Life Technologies) and 2 mM L-glutamine (Life Technologies). The authentication of all cell lines was performed by the company. The cells were cryopreserved and used within 6 months of resuscitation. Nlrp3 shRNA lentivirus and control shRNA were purchased from GeneChem (Shanghai, China).

U87 xenograft model and treatments

Four week old male nude mice (BALB/c-nude) are used to examine the tumorigenicity. A total of 100 μl (containing 5×10^6 U87 cells) cell solutions were subcutaneously injected into the right flank of the mice according to the method described previously [20]. The tumor volume was measured at different time points and the tumor volumes were calculated using the formula: $V = 0.52 \times [L \times W^2]$, (V = volume, L = length, and W = width). shRNA treatment: After the tumors reached a size of approximately 0.5 cm in diameter, the mice were treated by local tumor injection in blinding and randomization. IR treatment: IR of 2 Gy for five consecutive days was delivered to the tumor using a linear accelerator with a 4 MV nominal photon energy and a dose rate of 2.3 Gy/min.

Mouse GL261 model

6-week-old C57BL/6J mice (Harlan) were used for GL261 xenograft model. Ten thousand of GL261 cells were stereotactically injected into the left striatum into the following coordinates: 2 mm to the left of bregma and 4 mm below the surface of the skull at the coronal suture. After allowing 7 days for tumor establishment, IR of 2 Gy for five consecutive days was delivered to

Table 1. Aging-related genes regulated by Nlrp3 in hippocampus

ANP32B	acidic (leucine-rich) nuclear phosphoprotein 32 family, member B
ARPC1B	actin related protein 2/3 complex, subunit 1B, 41kDa
B2M	beta-2-microglobulin
CAST	calpastatin
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain
CTSL1	cathepsin L
GRIK1	glutamate receptor, ionotropic, kainate 1
GSN	gelsolin (amyloidosis, Finnish type)
HMGN2	high-mobility group nucleosomal binding domain 2
IGF1R	Insulin-like growth factor 1 receptor
LGMN	legumain
LYZ	lysozyme (renal amyloidosis)
MOBP	myelin-associated oligodendrocyte basic protein
NAP1L1	nucleosome assembly protein 1-like 1
NINJ2	ninjurin 2
PEX11A	Peroxisomal biogenesis factor 11A
PLEKHB1	pleckstrin homology domain containing, family B (evectins) member 1
PPAP2B	phosphatidic acid phosphatase type 2B
PRKCH	protein kinase C, eta
S100A6	S100 calcium binding protein A6 (calcyclin)
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
TYROBP	TYRO protein tyrosine kinase binding protein
ACAT1	acetyl-Coenzyme A acetyltransferase 1 (acetoacetyl Coenzyme A thiolase)
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1
FXYD7	FXYD domain containing ion transport regulator 7
GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)
H1F0	H1 histone family, member 0
Map1lc3a	microtubule-associated protein 1 light chain 3 alpha
NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4
RASGRF1	Ras protein-specific guanine nucleotide-releasing factor 1
SFXN3	sideroflexin 3
SSB	Sjogren syndrome antigen B (autoantigen La)
STMN2	stathmin-like 2
THY1	Thy-1 cell surface antigen 1

the tumor using a linear accelerator with a 4 MV nominal photon energy and a dose rate of 2.3 Gy/min. shRNA treatment: Mice were administered shRNA intravenously just before IR treatment. All experimental procedures involving animals were approved by the Animal Care and Use Committee of Shandong University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory.

Western blots

The cells were lysed and the protein concentrations were measured with the BCA Protein Assay (Pierce). 10 μg protein of each sample was separated in 10-15% SDS/PAGE gel, and then transferred to polyvinyldine fluoride membranes (Millipore), which was blocked in 5% fat free milk in TBST for one hour at room temperature. The membranes were then incubated with appropriate dilutions of specific primary antibodies overnight at 4°C and further visualized using chemiluminescence detection system. The primary antibodies for Nlrp3, caspase-1 and IL-1β antibody was purchased from Abcam, β-actin antibody was provided by Sigma.

Senescence-associated β-galactosidase activity

Senescent cells were analyzed using β-gal staining kit (Cell Signaling Technology) in accordance with the manufacturer's instructions as previously described [21].

Statistical analysis

Kaplan-Meier survival analysis was used to estimate the survival distributions, and the log-rank test was used to assess the statistical significance between groups. Cox proportional hazard models was fit to obtain HRs. Cox multivariate analysis was used to test whether the UM/DM group was an independent predictor for survival time. The Student's t test was used to determine differences in 2-group compari-

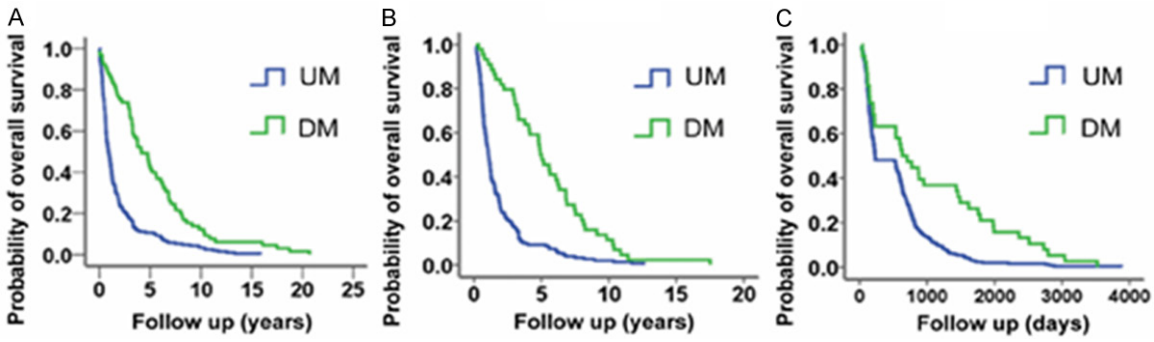

Figure 1. DM subtype had a superior overall survival compared with UM patients in (A) all of the glioma patient in GSE16011 cohort (B) radiotherapy-treated patients in GSE16011 cohort (C) GBM patients from TCGA.

Table 2. Multivariate Cox regression analysis in all the patients in GSE16011 cohort

Covariate	P	HR	95% CI
Gene signature	< 0.001	2.139	1.424-3.211
IDH1 mutation	0.093	1.322	0.954-1.832
Age at diagnosis	< 0.001	1.029	1.017-1.041
KPS	< 0.001	0.442	0.319-0.612
WHO	< 0.001		
WHO (I)	0.024	0.334	0.129-0.964
WHO (II)	0.048	0.595	0.355-0.996
WHO (III)	< 0.001	0.436	0.302-0.629

HR, hazard ratio; CI, confidence interval.

son. One-way ANOVA analysis was used to test for differences among at least 3 groups. All data are presented as mean ± standard error. P < 0.05 was considered statistically significant.

Results

Generation of gene signature regulated by Nlrp3 during aging

Previous studies reported that Nlrp3 inflammasome was an upstream target that controlled the process of aging in brain. Nearly all genes with age-dependent expression were regulated by Nlrp3 inflammasome in hippocampus of the old mouse [11]. Here, the genes that were regulated by Nlrp3 inflammasome in hippocampus of the old mouse have been derived by analyzing microarray data of GSE43034. To get the aging-related gene signature regulated by Nlrp3 inflammasome across mammalian species, microarray data of three rat and one rhesus hippocampal aging studies were analyzed with an overlap analysis to generate the aging-related gene signature in hippocampus. 101 genes were found to be commonly regulated in both species and 98 of the 101 genes were translated to human homolog gene symbols. NCBI Gene and NCBI HomoloGene databases were used to translate mouse gene probesets to human homolog gene symbols. At last, 34 of the 98 aging-related genes in hippocampus were found to be regulated by Nlrp3 inflammasome (**Table 1**).

Nlrp3 gene signature predicts glioma progression in radiotherapy-treated patients in GSE16011 data set

In order to understand the association between Nlrp3 gene signature and overall survival of patients with glioma, Hierarchical Clustering module in GenePattern was used to cluster the tumor samples from GSE16011 cohort into two major subtypes (UM and DM subtypes) based on the Nlrp3 gene signature expression. The gene signature were enriched in the UM group and down-regulated in the DM group.

We first performed Kaplan-Meier curve analysis in all patients in GSE16011 cohort. The results showed that DM subtype had a superior overall survival compared with UM patients (P < 0.001) (**Figure 1A**). We then performed Kaplan-Meier curve analysis in patients treated with radiotherapy. We found that in radiotherapy-treated patients, DM subtype also had a superior overall survival compared with UM patients (P < 0.001) (**Figure 1B**). Multivariate Cox regression analysis was performed to assess the prognostic value of Nlrp3 gene signature after adjusting for the known prognostic factors in GSE16011. The results showed that the gene signature was not only an independent predictor for survival time in all of the patient (**Table**

Table 3. Multivariate Cox regression analysis in radiotherapy-treated patients in GSE16011 cohort

Covariate	P	HR	95% CI
Gene signature	< 0.001	2.351	1.446-3.822
IDH1 mutation	0.127	1.340	0.917-2.015
Age at diagnosis	<0.001	1.033	1.018-1.048
KPS	0.002	0.563	0.388-0.815
WHO	0.002		
WHO (I)	0.454	0.459	0.060-3.522
WHO (II)	0.023	0.430	0.208-0.891
WHO (III)	< 0.001	0.470	0.309-0.714

HR, hazard ratio; CI, confidence interval.

Table 4. Multivariate Cox regression analysis in TCGA cohort

Covariate	P	HR	95% CI
Gene signature	0.005	1.539	1.349-1.834
Age at diagnosis	0.098	1.008	0.998-1.018
KPS	0.670	1.079	0.762-1.528
Additional chemotherapy	0.559	1.091	0.814-1.462

HR, hazard ratio; CI, confidence interval.

2), but also in radiotherapy-treated patients in GSE16011 cohort (**Table 3**).

Nlrp3 gene signature predicts glioma progression in radiotherapy-treated patients in TCGA cohort

Tumor samples from TCGA cohort were clustered into two major subtypes (UM and DM subtypes) based on the Nlrp3 gene signature expression as described above. In TCGA cohort, all patients had received IR therapy. Kaplan-Meier curve analysis was performed in the TCGA patients and the results showed that patients in DM subtype had a superior overall survival compared with UM patients ($P < 0.001$) (**Figure 1C**). In addition, multivariate Cox regression analysis showed that gene signature was an independent predictor of patient survival in adjuvant radiotherapy-treated patients (**Table 4**).

Radiation induces Nlrp3 inflammasome expression in mouse glioblastoma xenograft model

To investigate the effect of irradiation on Nlrp3 expression, a U87 xenograft mouse glioblastoma model was employed. Western blot analysis was conducted to determine the effect of irradiation treatment on levels of Nlrp3 inflammasome proteins. The results indicated that radiation treatment induced increased levels of Nlrp3, caspase-1 and IL-1β in vivo (**Figure 2A**).

Nlrp3 inhibition reduces tumor growth and IR-induced senescence in U87 glioma xenografts and prolongs the survival of mouse with GL261 GBM tumors following IR treatment

Previous studies showed that IR could induce senescence in human glioma cells [10, 11]. Cellular senescence could suppress cell proliferation; however, on the other hand, it also promoted tumor progression and recurrence through the release of senescence-associated secretory phenotypes (SASPs). We found that Nlrp3 inhibition significantly reduced the number of SA-β-gal-positive cells induced by IR treatment (**Figure 2B**). In addition, ablation of Nlrp3 reduced tumor growth and sensitized U87 tumor xenografts to IR in vivo (**Figure 2C**). Furthermore, we found that shNlrp3 treatment can prolong the survival of mouse with GL261 glioma tumors following IR treatment (**Figure 2D**).

Discussion

Despite the strong associations between patient age and glioma outcomes, the molecular link between aging and glioma progression is not fully understood. Recent studies reported that Nlrp3 inflammasome was an upstream target that controlled the process of brain aging. Nlrp3 inflammasome was also reported to play important roles in carcinogenesis and tumor progression [8-10]. Thus, Nlrp3 inflammasome may be a molecular link between aging and glioma progression.

From the clinical perspective, two patients diagnosed with glioma of identical stage and grade can have very different clinical outcomes. The tumors in different patients must be different at the molecular level. Recently, high throughput datasets has been widely used to to develop both prognostic and predictive biomarkers for patients with glioma. Gene signatures derived from high throughput datasets have been developed and validated to diagnose glioma, predict patient prognosis, and distinguish glioma grade at the molecular level [22-24]. In the present study, we generated a

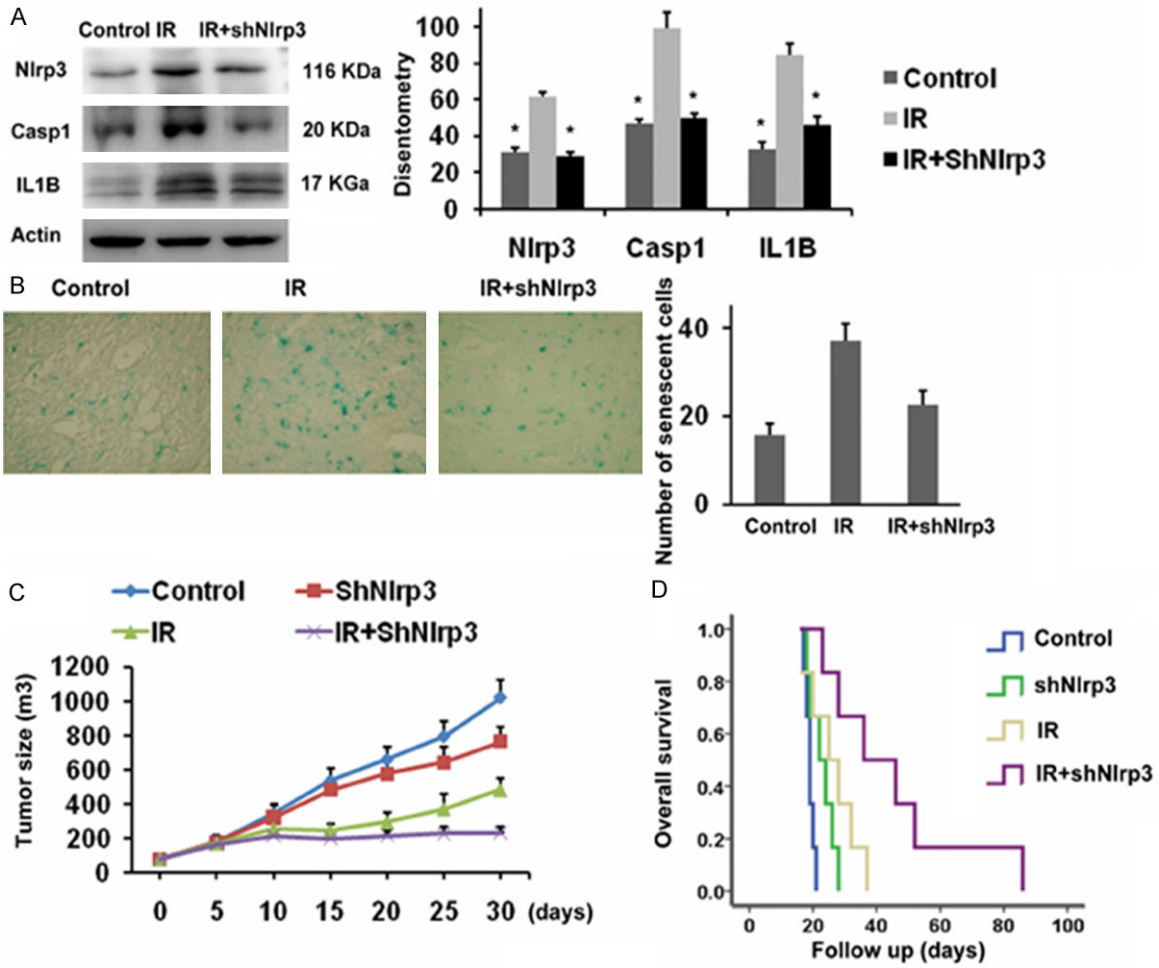

Figure 2. A. Radiation induces Nlrp3, caspase-1 and IL-1β expression in mouse glioma xenograft model, and local shNlrp3 treatment could reverse this effect. B. Local shNlrp3 treatment significantly reduced the number of SA-β-gal-positive cells in vivo after IR treatment. C. Local shNlrp3 treatment reduced tumor growth and sensitized U87 tumor xenografts to IR in vivo. D. shNlrp3 treatment prolonged the survival of mouse with GL261 glioma tumors following IR treatment. Error bars represent mean ± SD, $^{*}P < 0.05$.

aging-related gene signature that regulated by Nlrp3 in mouse hippocampus. We first examined the prognostic value of Nlrp3 gene signature in all glioma patients in GSE16011 cohort. We found that the DM subtype had a superior overall survival compared with UM patients.

Standard treatment for glioma patients is surgery followed by radiotherapy and adjuvant temozolomide (TMZ) chemotherapy, but tumors typically recur within 6 month of treatment [1]. This may be partly due to therapy resistance of glioma. Age has been identified as the most important prognostic factor for survival in glioma [2-4]. Resistance to chemoradiotherapy is a possible explanation for the comparatively worse outcome in the elderly patients with glioma [25, 26]. In addition, cell senescence, which has an intimate link with aging, has been demonstrated to play important roles in chemotherapy and radiotherapy resistance [27, 28]. We then examined the prognostic value of Nlrp3 gene signature in patients treated with radiotherapy in GSE16011 cohort. We found that in radiotherapy-treated patients, DM subtype had a superior overall survival compared with UM patients. Multivariate Cox regression analysis showed that the gene signature was an independent predictor of patient survival in radiotherapy-treated patients in GSE 16011. Similarly, in the TCGA data set, radiotherapy-treated patients assigned to DM group had an improved overall survival compared with UM group.

Previous studies showed that IR treatment can induce senescence in human glioma cells [12, 13]. Cellular senescence was an important

mechanism for preventing the proliferation of potential cancer cells, however, on the other hand, it also promoted tumor progression and recurrence through the release of senescence-associated secretory phenotypes (SASPs) [29, 30]. It was recently shown that chemokine released by senescent cells promoted the emergence, maintenance and migration of cancer stem-like cells [31]. In the present study, our findings showed that Nlrp3 inflammasome expression was induced by IR treatment in vivo, Nlrp3 inhibition reduced tumor growth in vivo and prolonged the survival of mouse following IR treatment. In addition, we found that shNlrp3 treatment reduced IR-induced senescence in U87 glioma xenografts. Our results above suggest that Nlrp3 inflammasome may be a potential therapeutic target to overcome radiation-resistance in glioma.

In conclusion, we demonstrated that Nlrp3 inflammasome was an important molecular link between brain aging and glioma progression and radiotherapy resistance. In addition, this Nlrp3 gene signature might served as a predictive biomarker in glioma patients and Nlrp3 inflammasome inhibition can provide a therapeutic strategy for radiation-resistant glioma.

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Disclosure of conflict of interest

None to declare.

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