Low miR-210 and *CASP8AP2* expression is associated with a poor outcome in pediatric acute lymphoblastic leukemia

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Received May 22, 2016; Accepted March 28, 2017

DOI: 10.3892/ol.2017.7229

Abstract. The prognostic significance of microRNA (miR)-210 and the caspase 8-associated protein 2 (*CASP8AP2*) gene in children with acute lymphoblastic leukemia (ALL) has been validated and *CASP8AP2* has been demonstrated as a target of miR-210. In the present study, the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine miR-210 and *CASP8AP2* expression in 91 children with ALL. Associations between gene expression levels and the prognostic value of combined detection of the two indicators were analyzed. Results from a receiver operating characteristic curve demonstrated that threshold values of miR-210 and *CASP8AP2* were 3.8243 and 0.4760, respectively. Although the expression of miR-210 and *CASP8AP2* were not associated at the mRNA level in pediatric ALL, combined detection of the two predicted ALL prognosis with an increased accuracy. Furthermore, an equation was devised including minimal residual disease at day 33 and expression of miR-210 and *CASP8AP2*, which may enable bone marrow relapse to be predicted more precisely compared with the current risk stratification.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of pediatric cancer, accounting for ~25% of all malignancies diagnosed in children <15 years (1). Although the outcome of childhood ALL has markedly improved with advancements in risk-adapted chemotherapy and supportive care (2), between 15 and 20% of patients eventually relapse (3) and recurrent ALL remains the primary obstacle in improving the cure rate and decreasing mortality (4).

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Key words: microRNA-210, caspase 8-associated protein 2, childhood acute lymphoblastic leukemia, relapse, minimal residual disease

Minimal residual disease (MRD) in the early stages of treatment has been widely recognized as one of the most powerful prognostic indicators. However, not all patients with positive MRD relapse and certain patients with negative MRD may relapse. To avoid inadequate therapy for high-risk patients and over-treatment for low-risk patients (5), novel prognostic indicators are urgently required for risk refinement.

An independent study has indicated that microRNA (miR)-210 is consistently and predominantly upregulated in hypoxic states (6). By acting on target genes, miR-210 is involved in a range of physiological and pathological processes (7-9). In our previous study (10), it was demonstrated that miR-210 is an independent prognostic factor for pediatric ALL and that low miR-210 expression (threshold value, 3.8243) is a good predictor for relapse and induction failure in childhood ALL.

Caspase 8-associated protein 2 (CASP8AP2), a component of Cajal bodies, is an essential factor in regulating histone gene transcription, apoptosis and S phase progress (11-13). Flotho *et al* (14) demonstrated that decreased *CASP8AP2* expression is markedly associated with increased rates of MRD and hematological relapse. Kim *et al* (15) demonstrated that *CASP8AP2* is a target of miR-210 in bone marrow-derived mesenchymal stem cells. However, an association between expression levels in ALL cells was not identified.

In the present study, the clinical significance of *CASP8AP2* and the association between *CASP8AP2* and miR-210 was analyzed. In addition, the prognostic value of combined detection of miR-210 and *CASP8AP2* expression was determined.

Materials and methods

Patients and treatment. Between March 2008 and July 2010, 203 children with newly diagnosed ALL were enrolled in the Chinese Children's Leukemia Group (CCLG)-ALL 2008 protocol at Beijing Children's Hospital. Criteria for patient inclusion were ≥70% leukemic cells in diagnostic bone marrow (BM) samples (16), treatment according to the CCLG-ALL 2008 protocol (17) and sufficient BM sample for total RNA/microRNA (miRNA) extraction. A total of 112 children with ALL were excluded from analysis.

On the basis of these criteria, 91 patients (median age, 5 years; range, 1.0-14 years) were included in the present study (57 boys and 34 girls), with 81 cases of B cell precursor ALL (BCP-ALL) and 10 cases of T cell ALL (T-ALL). The median follow-up time was 37.2 months (range, 1.0-50.0 months). A total of 11 patients suffered from BM relapse or induction failure and all succumbed between 3 and 11 months after relapse or induction failure. A further 2 patients succumbed due to severe infection and the remaining 78 patients were in continuous complete remission (CCR). BM samples obtained from 5 ALL patients in CCR for >5 years (control group) were used as calibrators (18).

The CCLG-ALL 2008 protocol was approved by the Beijing Children's Hospital Institutional Ethics Committee and written informed consent was obtained by the patients' guardians.

miRNA isolation, reverse transcription and determination of miR-210 expression. Total miRNA was extracted using the mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol as in our previous study (10). Collected miRNA was stored at -80˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine miR-210 expression. Laboratory procedures and experimental details were as described in a previous study (10).

RNA isolation and cDNA synthesis. Mononucleated BM cells were isolated by Ficoll-Hypaque density-gradient centrifugation (MD Pacific Biotechnology Co., Ltd., Tianjin, China) and stored at -80˚C until use. Total RNA was extracted within 2 weeks using TRIzol reagent according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). mRNAs were reverse-transcribed into cDNAs using random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

Quantitative analysis of CASP8AP2 expression. *CASP8AP2* expression was detected using RT-qPCR. The primers and TaqMan probes, which were designed using Primer Express (version 3.0; Applied Biosystems; Thermo Fisher Scientific, Inc.) are listed in Table I. A TaqMan probe of the Abelson (*ABL*) gene was used as an internal control and associated primer sequences are described previously (19).

The reaction mixture contained TaqMan Master ROX mix (6.25 μl), 10 pmol each primer, 2.5 pmol probe, cDNA template (1 μl) and deionized water to a total volume of 12.5 μl. The reaction was performed at 95˚C for 10 min, followed by 50 cycles of 15 sec at 95˚C and 1 min at 60˚C on a 7500 Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each sample was detected in triplicate. *CASP8AP2* expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and are presented as fold change compared with the control group (20).

Detection of MRD. MRD monitoring was performed by qPCR at the end of induction therapy (day 33). Patient-specific immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements, including IgH, IgK, IgL, Kde, TCRB, TCRG and TCRD, were used as qPCR targets for quantitative assessment of MRD. Detection methods were as previously described (21).

Table I. Sequences of primers and probe for *CASP8AP2* and *ABL*.

Genes	Sequences of primers and probe (5'-3')
CASP8AP2	CACTTGCCACTTCTACAAGTC (sense) TGGCGGCTAAATATGCAAATG (antisense) FAM-TGTCAGAAAAGAGGGCCATCATTTAAA-TAMRA (probe)
ABL	ATCCAAGAAGGGGCTGTCC (sense) CCAACGAGCGGCTTCAC (antisense) FAM-CCTTCAGCGGCCAGTAGCATC-TGA-TAMRA (probe)

CASP8AP2, caspase 8-associated protein 2; *ABL*, Abelson murine leukemia viral oncogene homolog 1; FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamine.

Statistical analysis. A receiver operating characteristic (ROC) curve was used to assess the predictive value of *CASP8AP2* expression for relapse. Relapse-free survival (RFS) was defined as the time from the diagnostic date through the date of relapse at any site. Event-free survival (EFS) was estimated from the date of diagnosis to the date of induction failure, relapse, second tumor or mortality. Overall survival (OS) was defined as the time between diagnosis and mortality or last contact with the patient in CCR. Kaplan-Meier estimator survival analysis was used to determine the differences in RFS, EFS and OS. Spearman's correlation was used to determine the association between *CASP8AP2* and miR-210. A Cox's proportional hazards model was utilized to determine an equation for assessment of the risk of bone marrow relapse. All analyses were performed with SPSS (version 16.0; SPSS, Inc., Chicago, IL, USA) for Microsoft Windows. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical value of CASP8AP2 expression. No statistically significant differences were identified between the included and excluded patients regarding age (P=0.752), sex (P=0.313), immunophenotype (P=0.083), transcription factor ETV6-AML (P=0.143), breakpoint cluster region-*ABL* (P=0.725), transcription factor 3-PBX homeobox 1 (P=0.902), myeloid/lymphoid or mixed-lineage leukemia rearrangement (P=0.837) and central nervous system (CNS) involvement (P=0.110).

Median *CASP8AP2* expression in the 91 children evaluated was 0.6591 (range, 0.21-2.05). According to ROC curve analysis, the optimal threshold value for *CASP8AP2* expression was 0.4760 [area under the curve (AUC), 0.865; 95% confidence interval, 0.725-1.006; P<0.001] with a sensitivity and specificity of 0.850 and 0.818, respectively.

Using this threshold value, the 91 patients were divided into low (n=21) and high (n=70) expression groups. The relapse rate

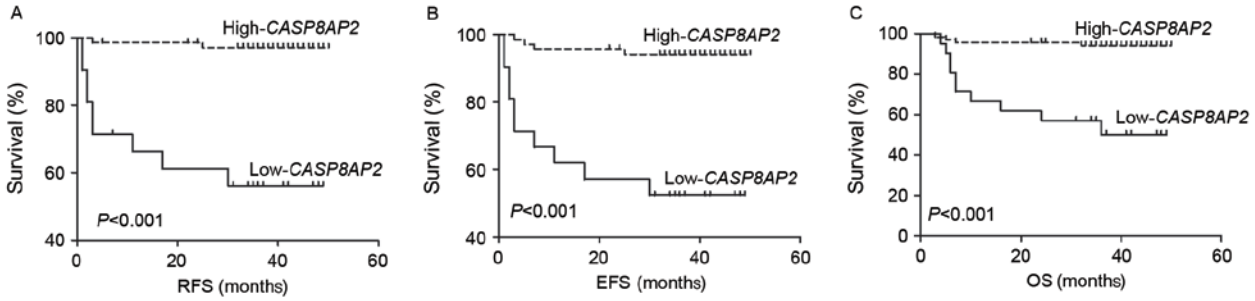

Figure 1. Kaplan-Meier estimates of (A) RFS, (B) EFS and (C) OS for children with ALL with increased and decreased expression of *CASP8AP2*. RFS, relapse-free survival; EFS, event-free survival; OS, overall survival; ALL, acute lymphoblastic leukemia; *CASP8AP2*, caspase 8-associated protein 2.

in the low-*CASP8AP2* group (9/21; 42.8%) was significantly increased compared with that of the high-*CASP8AP2* group (2/70; 2.9%; P<0.001). The low-*CASP8AP2* group exhibited decreased RFS (log-rank: P<0.001), EFS (log-rank: P<0.001) and OS (log-rank: P=0.005) compared with the high-*CASP8AP2* group (Fig. 1A-C). The results of the present study indicated that *CASP8AP2* expression in patients with newly diagnosed ALL is a valuable marker for predicting relapse.

Association between miR-210 and CASP8AP2 expression. Using the threshold value (3.8243) determined in our previous study, the cohort of 91 patients was divided into low (n=41) and high (n=50) expression groups. No association was identified between *CASP8AP2* and miR-210 expression in these groups, regardless of continuous or grouped values (P>0.05). Associations between *CASP8AP2*, miR-210 and clinical characteristics are presented in Table II.

Prognostic relevance of miR-210 and CASP8AP2 expression. miR-210 and *CASP8AP2* expression are known prognostic indicators in pediatric ALL which prompted the determination in the present study of the efficacy of combining miR-210 and *CASP8AP2* expression to predict relapse. The 91 patients were stratified into four groups according to miR-210 and *CASP8AP2* expression. Of the 40 cases in the double high-expression group (miR-210^{high}/*CASP8AP2*^{high}), none of the patients relapsed, with 3-year EFS and OS values of 93.1±9.9 and 95.7±0.3%, respectively. A total of 11 patients with double low-expression of the two genes (miR-210^{low}/*CASP8AP2*^{low}) exhibited the poorest outcomes with 3-year RFS, EFS and OS values of 27.3±13.4, 27.3±13.4 and 36.4±14.5%, respectively. No statistically significant difference was observed in prognosis between patients with single low-expression of the two genes (miR-210^{low}/*CASP8AP2*^{high}, n=30; miR-210^{high}/*CASP8AP2*^{low}, n=10) with 3-year RFS, EFS and OS values as follows: 91.7±5.6 vs. 88.9±10.5%; P=0.830; 91.7±5.6 vs. 80.0±12.6%; P=0.338; and 91.3±5.9 vs. 80.0±12.6%; P=0.351, respectively. The two subgroups were combined into a single group (n=40) and an intermediate prognosis was determined with 3-year RFS, EFS and OS values of 91.0±5.0, 88.0±5.6 and 83.3±6.9%, respectively (Fig. 2A-C). The results of the present study indicate that combined detection of miR-210 and *CASP8AP2* expression may accurately predict ALL relapse.

Estimation of relapse risk based on clinical features, miR-210 and CASP8AP2 expression. In COX regression analysis, white blood cell counts, MRD at day 33, prednisone response, CNS involvement, *BCR-ABL1, TEL-AML, E2A-PBX1, MLL* rearrangement, and miR-210 and *CASP8AP2* expression were considered covariates. Results of the present study indicated that MRD at day 33, miR-210 and *CASP8AP2* expression are all independent prognostic indicators (Table III). On the basis of the final Cox's proportional hazards model for RFS, an equation, composed of the three factors, was devised to estimate the risk of relapse as follows: Risk index =3.393x MRD-3.549x miR-210-2.855x *CASP8AP2*

In the aforementioned equation, MRD represents MRD at day 33 (1 for MRD $<10^{-4}$ and 2 for MRD $\geq10^{-4}$), miR-210 represents miR-210 expression levels (1 for low-miR-210 and 2 for high-miR-210) and *CASP8AP2* represents *CASP8AP2* expression levels (1 for low-*CASP8AP2* and 2 for high-*CASP8AP2*). The predictive value of this algorithm was tested using an ROC curve. The AUC was 0.965 (P<0.001), which was improved compared with miR-210 and CASP8AP2 expression or clinical risk stratification alone (0.789, 0.865 and 0.841, respectively; Fig. 3), indicating that combined assessment of miR-210 and *CASP8AP2* expression may identify patients at increased risk of relapse.

Discussion

In the present study, the AUC of the ROC curve of the current clinical risk stratification was 0.841, suggesting that improvement is required. The prognostic value of miR-210 and *CASP8AP2* has been demonstrated in previous studies, and *CASP8AP2* has been demonstrated as a target of miR-210 in stem cells. The present study evaluated the association between miR-210 and *CASP8AP2* in pediatric ALL at the mRNA level and explored the prognostic significance of joint detection. The results of the present study identified that decreased miR-210 or *CASP8AP2* expression in newly diagnosed ALL patient BM samples was associated with increased MRD, increased BM relapse rate and poor RFS, EFS and OS. Multivariate analyses indicated that miR-210 and *CASP8AP2* expression are independent prognostic factors following adjustment for other risk factors. Combined assessment of miR-210 and *CASP8AP2* expression is considered an improved method, compared with a single assessment or the current clinical risk stratification, in identifying patients at increased risk of relapse. Furthermore, an equation was devised for estimating bone marrow relapse risk, based on MRD at day 33 and miR-210 and *CASP8AP2* expression. As

Table II. Association of miR-210 and *CASP8AP2* expression with clinical characteristics.

		miR-210 expression			*CASP8AP2* expression		
Characteristic	Patients (n=91)	Low	High	P-value	Low	High	P-value
Age, years				0.083			0.402
1-9	73	25	48		16	57	
>10	18	10	8	0.02	5	13	
Sex				0.184			0.434
Male	57	27	30	0.056	14	43	
Female	34	8	26		7	27	
WBC count, cells/l				0.128			0.582
$<50x10^9$	61	21	40		14	47	
$\geq 50x10^9$	30	14	16	0.5	7	23	
MRD (at day 33)							0.001
Positive	67	22	45	0.624	9	58	
Negative	24	13	11		12	12	
Immunophenotype							0.274
BCP-ALL	81	29	52	0.151	20	61	
T-ALL	10	6	4		1	9	
Prednisone response				0.009			0.227
Good	87	33	54		19	68	
Poor	4	2	2	0.385	2	2	
CNS involvement							0.41
No	89	34	55		20	69	
Yes	2	1	1		1	1	
Fusion genes							
BCR-ABL							0.002
Positive	6	4	2		5	1	
Negative	85	31	54		16	69	
TEL-AML1							0.016
Positive	27	5	34		2	25	
Negative	64	30	22		19	45	
MLL-AF4							0.231
Positive	1	1	0		1	0	
Negative	90	34	56		20	70	0.079
E2A-PBX1				0.366			
Positive	8	4	4		4	4	
Negative	83	31	52		17	66	

miR, microRNA; *CASP8AP2*, caspase 8-associated protein 2; WBC, white blood cell; MRD, minimal residual disease; BCP, B cell precursor; ALL, acute lymphoblastic leukemia; CNS, central nervous system; *BCR-ABL*, breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 fusion gene; *TEL-AML1*, ETS variant 6-acute myeloid leukemia 1 fusion gene; *MLL-AF4*, myeloid/lymphoid or mixed-lineage leukemia-ALL 1 fused gene on chromosome 4 fusion gene; *E2A-PBX1*, transcription factor 3-PBX homeobox 1 fusion gene.

expected, the equation predicted treatment outcome more precisely than clinical risk stratification alone.

Functioning as a hypoxamir, miR-210 participates in regulation of a number of physiological and pathological processes including cell survival, proliferation, differentiation, apoptosis and development (7-9). A previous study has indicated that leukemic bone marrow is likely in a hypoxic microenvironment at the initial diagnosis, due to the increased proliferation and oxygen consumption of leukemic cells (22). This is consistent with the results of the present study indicating that increased miR-210 expression is prevalent in BM samples at initial diagnosis. In our previous study, the prognostic significance of decreased miR-210 expression in pediatric ALL was demonstrated (10). However, Zhang *et al* (23) identified that miR-210 expression in a high-risk group (HR) was significantly increased, compared with that in intermediate risk (IR)

Table III. Prognostic significance of miR-210 and *CASP8AP2* expression levels and other common clinical features analyzed by Cox's proportional hazards model.

			95% confidence intervals for HR	
Features	Hazard ratio (HR)	P-value	Lower	Upper
miR-210	0.029	0.012	0.002	0.461
CASP8AP2	0.058	0.015	0.006	0.575
MRD (at day 33)	29.742	0.026	1.498	590.316
Prednisone response	1.352	0.796	0.138	13.259
CNS involvement	4.246	0.450	0.100	180.757
BCR-ABL	0.467	0.534	0.042	5.147
TEL-AML	0.310	0.410	0.019	5.043
E2A-PBX1	3.151	0.436	0.176	56.447
*MLL r*earrangements	1.914	0.658	0.108	33.988

miR, microRNA; *CASP8AP2*, caspase 8-associated protein 2; MRD, minimal residual disease; CNS, central nervous system; *BCR-ABL*, breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 fusion gene; *TEL-AML1*, ETS variant 6-acute myeloid leukemia 1 fusion gene; *E2A-PBX1*, transcription factor 3-PBX homeobox 1 fusion gene; *MLL*, myeloid/lymphoid or mixed-lineage leukemia gene.

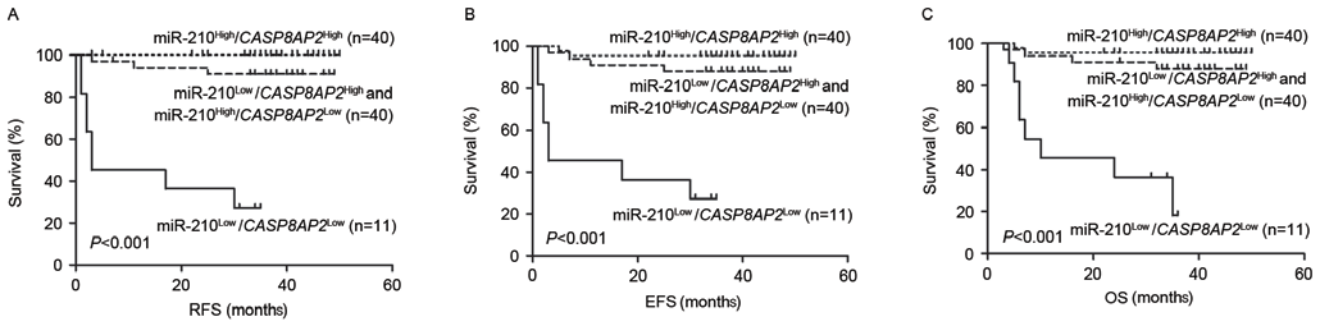

Figure 2. (A) Relapse-free survival, (B) event-free survival and (C) overall survival of children with acute lymphoblastic leukemia stratified by combined assessment of miR-210 and *CASP8AP2* expression. miR, microRNA; *CASP8AP2*, caspase 8-associated protein 2.

or standard risk (SR) groups, indicating that increased miR-210 expression is associated with an poorer outcome in pediatric ALL, which is in contrast with the results of the present study. The reasons for the conflicting results may be a substantial difference in the risk classification between the two groups. Zhang *et al* (23) conducted a study in which the proportion of HR patients was increased compared with that in the present study (36.7%, 18/49 vs. 17.5%, 16/91, respectively). In addition, Zhang *et al* (23) extracted total miRNAs using TRIzol reagent and detected miRNA levels using an miRNA chip, whereas the present study used mirVana miRNA Isolation kit, TaqMan MicroRNA Assay and RT-qPCR.

Kim *et al* (15) demonstrated that *CASP8AP2* is the target of miR-210 in bone marrow-derived mesenchymal stem cells. However, the present study did not identify an association between these factors; this may be due to the fact that miRNAs regulate gene expression post-transcriptionally which failed to demonstrate a negative association at the mRNA level (24). The complex regulatory network, including miR-210 and its target genes, varies in distinct cell types and further studies are required to explore additional possible associations. Target genes of miR-210 in pediatric ALL have not been studied and the underlying molecular mechanisms of decreased miR-210 expression associated with a poor prognosis remain unclear. Additional studies are required to elucidate the underlying molecular mechanisms of poor prognosis linked to decreased

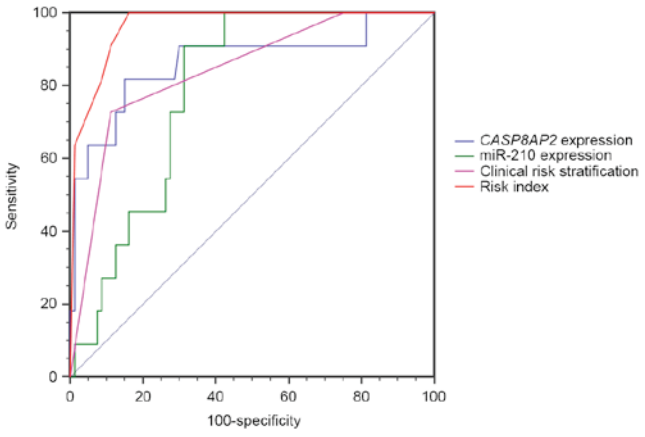

Figure 3. Comparison of the predictive value for relapse of miR-210 or *CASP8AP2* expression, current clinical risk stratification and the novel equation. The area under the curve was 0.789, 0.865, 0.841 and 0.965, respectively. miR, microRNA; *CASP8AP2*, caspase 8-associated protein 2.

miR-210 and *CASP8AP2* expression in pediatric ALL. Studies of the role that these indicators serve in drug resistance may provide insight into their prognostic value in treating pediatric ALL.

Acknowledgements

The present study was supported by the Priming Scientific Research Foundation for the Junior Researcher in Beijing Tongren Hospital, Capital Medical University (grant no. 2014-YJJ-ZZL-011), the Key Scientific Research Training Fund of Beijing Tongren Hospital, Capital Medical University (grant no. 2015-YJJ-GGL-009).

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