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Association of *ABCB1*, *ABCC5* and *xanthine oxidase* genetic polymorphisms with methotrexate adverse reactions in Mexican pediatric patients with ALL

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is one of the most frequent oncological disorders in pediatric populations. To date, the drug of choice for the treatment of ALL is methotrexate, a drug associated with a high risk of adverse reactions (ADRs). The xanthine oxidase (*XO*) polymorphisms, *1936A>G* and *2107A>G*, as well as the polymorphic variants derived from ATP-binding cassette transporter gene subfamilies, *ABCB1* and *ABCC5*, of drug resistant codifying genes, are implicated as precursors of drug-related neurologic, hepatic, and renal toxicities. Our aim was to determine whether the mentioned polymorphisms are risk or protective factors for the development of adverse reactions by methotrexate in our pediatric population with ALL.

Materials and methods: A total of 35 Mexican children from Centro Estatal de Cancerología-Durango, Mexico, with ALL and the previously noted polymorphisms as determined qPCR were studied. At the same time, a 12-month drug monitoring program was conducted in accordance with WHO-PAHO guidelines for pharmacovigilance.

Results: The *ABCB11936A>G* and *2107A>G* and *ABCC5 3414+434A>C* polymorphisms were not associated with methotrexate ADRs. Single nucleotide polymorphisms (SNPs) of *ABCB1* 1236C>T (OR 0.19, 95% CI: 0.03–0.9, $p<0.05$) and *ABCC5 3933+313T>C* (OR 0.12, 95% CI: 0.027–0.58, $p<0.05$) were associated with methotrexate ADRs.

Conclusions: SNPs *1236C>T* of *ABCB1* and *ABCC5 3933+313T>C* are not associated with the development of typical ADRs by methotrexate, rather, they showed a protective factor for myelosuppression in the studied sick population.

Keywords: ATP-binding cassette transporter gene; genetic polymorphisms; methotrexate; pharmacovigilance; precursor cell lymphoblastic leukemia-lymphoma/*drug therapy/genetics; xanthine oxidase (*XO*).

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Introduction

Acute lymphoblastic leukemia (ALL) is a common worldwide hematologic malignancy of public health importance. Between 80% and 85% of pediatric patients with a diagnosis of leukemia have ALL [1]. In northeast Mexico, the incidence and mortality of ALL is frequent [2]. The influence of proteins that determine drug resistance has been recently highlighted. The main function of these proteins, known as multi-drug resistance proteins (MDR1 and MRP5), is to enable efflux of pharmacologically active molecules from tumor cells (e.g. lymphoblasts) [3–5].

The over-expression of *ABC* transporters in tumor cells is a primary mechanism of resistance to chemotherapeutic drugs [6]. Since the functional characterization of MRP5, its importance has been identified in the resistance to anti-neoplastic and anti-HIV drugs [7]. The antifolate mechanism of action of MTX compromises the gastrointestinal tract and bone marrow, thereby increasing the risk of toxicity [8, 9]. Liu et al. [10] found that the –24T allele of the *ABCC2* gene is associated with the severity of MTX toxicity via its resultant long duration of high drug concentration. This finding suggests that individualization of methotrexate dosing might result in less toxicity.

Meanwhile, xanthine oxidase (*XO*) is an enzyme that metabolizes purines and transforms them into uric acid. Kudo et al. [11] conducted genotyping studies and found that certain polymorphisms can reduce enzyme function in approximately 64.7% of Caucasians. *XO* activity can be reduced by some genotypes or inhibition by allopurinol or MTX. Reduced *XO* activity results in a decrease in metabolism and the elimination of natural and synthetic purines (e.g. 6-mercaptopurine, oncologic treatments), which could be a factor in the development of ADRs [12].

Several researchers have found significant association between the polymorphisms of ABCB1 with changes in efficiency and toxic responses by MTX in pediatric population with ALL [13–14]. In contrast, reports about the role of ABCC5 genetic polymorphisms in the onset adverse drug reactions (ADRs) by MTX in Mexican pediatric patients with ALL are limited. Therefore, the main aim of the current study was to determine whether genetic polymorphisms of the membrane transport proteins *ABCB1* and *ABCC5*, or *XO* are associated with the development of ADRs by MTX in Mexican pediatric patients with ALL.

Materials and methods

General study design

This was an observational, prospective, association study. A total of 35 pediatric patients were assessed in our study. All children satisfied the following criteria of inclusion: being in pediatric age, both genders, having diagnostic of low and intermediated risk of ALL, agree on the Franco-American British Association criteria [15], giving signed informed consent by tutors, and being in maintenance phase with treatment of low-dose of MTX. Our patients were admitted between August 2012 to November 2013 at the Hematology-Oncology Unit, Durango State Cancer Center (Centro Estatal de Cancerología, CECAN), Durango, Mexico. This research was approved by the CECAN Ethic and Research Committee, Durango, Mexico, in accordance with the Helsinki Declaration and Mexican general health law. Each patient received chemotherapy courses, in accordance with the St. Jude TOTAL XV protocol [16]. The study focused on the maintenance stage, which consisted of 120 weeks during MTX administration, and was carried out for low risk and moderate patients. We monitored 538 courses of chemotherapy in low-risk patients. The observations of courses of chemotherapy was evaluated in three stages. The first stage consisted of a single dose of MTX (25–50 mg/m^2) i.m. with 6-mercaptopurine (6-MP) oral 75 mg/m^2/dose once a day, vincristine (2 mg/m^2/dose) and dexamethasone (8 mg/m^2/day for 5 days). For the second stage, the children received a single dose MTX (25–50 mg/m^2) i.m. in the morning and 6-MP 75 mg/m^2/dose in the night. In the last stage, we reviewed courses without administration of MTX for reach homogeneity of the results. For this research, the second phase focuses on reducing interferences and drug interactions.

Genotyping

DNA extraction was carried out using a commercial kit (Macherey-Nagel®, Germany). DNA integrity and purity were evaluated by horizontal electrophoresis in 1% agarose gel stained with ethidium bromide. This was followed by quantification in a spectrophotometer (Nanodrop®, Thermoscientific, USA). Determination of *XO, ABCB1*, and *ABCC5* allelic variants was done using real-time polymerized chain reaction (qPCR) (Applied Biosystems StepOne™, USA). The catheter (Taqman®) and assays used are presented in Table 1.

Pharmacovigilance

The detection of ADRs by MTX and the documentation of simultaneously prescribed drugs within the therapeutic plan were carried out in accordance with the modified algorithm of Naranjo et al. [17], The Federal Commission for Protection against Health Risks (COFEPRIS), and the World Health Organization (WHO) [18–20].The considered variables were age, sex, body weight, height, nutritional status, leukocyte count, leukoblasts count, hemoglobin, hematocrit, erythrocytes, thrombocytes, hepatic function tests, and renal function tests. To evaluate MTX toxicity in each treatment course, the levels of myelosuppression, hepatotoxicity, mucositis, neurotoxicity, and nephrotoxicity were determined in the maintenance phase. The erythrocyte count, leukocyte count, hemoglobin value, hematocrit, and platelet were also considered. Hepatotoxicity was determined with bilirubin values (direct, indirect, and total), transaminases, jaundice, choluria, hepatomegaly, and gastrointestinal reactions, such as nausea, emesis, diarrhea, and flatulence. Oral mucositis was evaluated in accordance with WHO criteria and the National Cancer Institute (NCI) adverse event terminology [21]. Neurotoxicity was evaluated based on seizure activity, ataxia, apraxia, paresis, paralysis, level of consciousness, mood, and visual disorders. Finally, nephrotoxicity was assessed by oliguria, edema, dysuria, proteinuria, plasma urea, serum creatinine, and reduction in creatinine clearance.

Statistical analyses

The Hardy-Weinberg equilibrium and binding disequilibrium analyses were done from the expected and observed genotypic and allelic frequencies in the study population. SNPStats (Spain) online software was used [22].

Table 1: Characteristics of the studied single nucleotide polymorphisms (SNPs) with their National Center for Biotechnology Information (NCBI) and by Applied Biosystem references.

SNPs	Locus	Id NCBI	Sequence	Id Catheter Applied Biosystems®
ABCB1(MDR1) *c1236T>C*	CCr7	rs1128503	GCCCACTCTGCACCTTCAGGTTCAG[A/G] CCCTTCAAGATCTACCAGGACGAGT	C_7586662_10
ABCC5(MRP5) *3414+434A>C*	Cr3	rs9838667	TCAATCACCCCTAGGGGCTAGAAGG[G/T] TTTATGTATATAACAGTATTGGGAA	C_ 26061042_10
ABCC5(MRP5) *3933+313T>C*	Cr3	rs3792585	GCCACGTTATATATCTTTTGCGTAT[A/G] TGTGGACGCTTCAAAGTATGTTACA	C_1738056_10
Xanthine Oxidase 1936A>G	Cr2	rs17323235	GTCTCATCATTACAAATTCCAGTTA[C/T] GTTACTCCCAGGAACATCATCAGCG	C_25603303_20
Xanthine Oxidase 2107A>G	Cr2	rs1701368	GGTCCATAAAAGGAGTTGTTCTTTA[C/T] AGCATCCTGAGGATCACAAAGAAGT	C_25472962_20

The polymorphism frequencies and ADRs during the MTX maintenance phase were obtained. The associations of the *XO*, *ABCB1*, and *ABCC5* genetic polymorphisms with ADRs by MTX were assessed by analysis of risk, expressed as the coefficient of the odds ratio (OR) and considered significant when the p-value was <0.05 and 95% confidence interval (95%CI) was >1. Test canonical regression was evaluated to search associations between all variables. SAS version 9.0 (USA, 2002) and Statistic version 7 (USA, 2004) were used.

Results

Genotyping results

A total of 35 patients with a diagnosis of ALL were studied. Demographic data are displayed in Table 2. Table 3 shows the genotype and allele frequencies. Of the 35 patients, 21 were homozygous for the *ABCB1* variant *1236C>T* that encodes for multidrug transporter MDR1, four subjects were heterozygotes (HT), and 10 were classified as wild-type homozygotes (WT). Moreover, *3414+434A>C* and *3933+313T>C ABCC5* polymorphisms encoding for MRP5 were studied. Of the 35 children studied, eight were HM, four were HT, and 23 were WT for the *3414+434A>C* polymorphism. In addition, 15 were HT, six were HM, and 14 were WT for the *3933+313T>C* polymorphism.

For the *XO* 1936A>G polymorphism, 21 subjects were HT, six were HM, and eight were WT. For the *2107A>G* polymorphism, 21 subjects were HM, 15 were WT, and none were HT. A Hardy-Weinberg equilibrium analysis revealed non-existence in the polymorphisms *ABCB1* (*1236C>T*), *3414+434A>C* of *ABCC5* and *XO* (*2107A>G*) (Table 3). Linkage disequilibrium analysis of the *ABCC5* polymorphisms showed the flowing value D´=0.02 (p=0.88) and *XO* SNPs and D´=0.15 (p=0.30) for *XO* SNPs. The findings demonstrate non-independence in the genetic co-segregation.

Table 2: Demographic data and posology clinical biochemical tests for 35 pediatric oncology patients included in the study.

Variables	Median (range)
Age, years	6.3 (2.5–18.1)
Sex, male/female	22 (0.62)/13 (0.38)
Body weight, kg	18.9 (12.3–55.1)
Height, m	1.11 (0.91–1.71)
Body surface, m^2	0.71 (0.50–1.7)
Body mass index, kg/m^2	15.11 (12.4–28.1)
Methotrexate i.m. average dose	45.2 mg/m^2
Dosing interval	Week 2 and 3
Hemoglobin, g/dL	12.05 (7–16.8)
Hematocrit, %	36.1 (28.2–47.0)
Erythrocyte count, $cell/mm^3$	3.59×10^6 ($2.85–5.89\times10^6$)
Leukocyte count, $cell/mm^3$	3220 (1079–13600)
Platelet count, $cell/mm^3$	187×10^3 ($41.1–378\times10^3$)
AST, U/L	44.5 (0.4–149)
ALT, U/L	61 (0.3–206)
LDH, U/L	644 (352–1496)
Uric acid, mg/dL	3.4 (2–4.7)
Plasma urea, mg/dL	18 (8–34)
Serum creatinine, mg/dL	0.5 (0.3–0.7)
Alkaline phosphatase, U/L	249 (58–317)

Pharmacovigilance results

The association between the risk of developed ADRs with the genotype of the 35 pediatric patients is shown in Table 4. As can be seen, *ABCB1 1236T*>C and *3933+313T>C* polymorphisms showed protective effect for myelosupression with OR of 0.19(0.03–0.93) and 0.125 (0.027–0.579), respectively.

Table 3: Genotype and allelic frequency polymorphisms regulating drug resistance proteins (*ABCB1* and *ABCC5*) and xanthine oxidase (*XO*).

Gene	Polymorphism (SNP)	Frequencies					
		Genotypes[a]			Alleles[b]		
		WT	HT	HM	*p*	*q*	Hardy-Weinberg
ABCB1(MDR1)	*1236C>T*	10C/C	4C/T	21T/T	24	46	p<0.0001
n=35		(0.29)	(0.11)	(0.60)	(0.43)	(0.57)	
ABCC5 (MRP5)	*3414+434A>C*	23A/A	4A/C	8C/C	50	20	p<0.0001
n=35		(0.66)	(0.11)	(0.23)	(0.71)	(0.29)	
ABCC5 (MRP5)	*3933+313T>C*	14T/T	15T/C	6C/C	43	27	p=0.72
n=35		(0.40)	(0.43)	(0.17)	(0.61)	(0.39)	
XO	*1936A>G*	8A/A	21A/ G	6G/G	37	33	p=0.32
n=35		(0.23)	(0.60)	(0.17)	(0.53)	(0.47)	
XO	*2107A>G*	15A/A	0A /G	20G/G	30	40	p<0.0001
n=35		(0.43)	(0.00)	(0.57)	(0.43)	(0.57)	

WT, Wild type; HT, heterozygote; HM, homozygote mutated or variant; *XO*, xanthine oxidase; SNP, single nucleotide polymorphism. [a]Number of patients and their respective proportion in parenthesis, [b]Hardy-Weinberg notation for allelic frequencies where *p* represents the value of wild-type allelic frequency and *q* mutated allele p<0.05.

Table 4: Frequencies of pediatric patients who had adverse reactions by genetic polymorphism.

Polymorphism	Genotype	Myelo suppression[a]		Hepato toxicity		Nephro toxicity		Neuro toxicity		Mucositis	
		Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
ABCB1	*CC*	7	3	22	9	1	9	2	8	2	8
1236C>T	*CT+TT*	8	18	3	1	2	23	3	2	9	16
	p-Value		0.033		0.440		0.458		0.318		0.22
	OR (CI)	0.19 (0.03–0.93)		1.22 (0.11–13.43)		0.78 (0.06–9.74)		0.54 (0.07–3.89)		2.25 (0.39–12.97)	
ABCC5	*AA*	10	13	2	21	1	22	2	21	9	14
3414+434A>C	*AC+CC*	5	7	2	10	2	10	3	9	2	10
	p-Value		0.229		0.318		0.232		0.1715		0.129
	OR(CI)	0.93 (0.23–3.82)		2.10 (0.26–17.14)		4.40 (0.36–54.37)		3.50 (0.49–24.66)		0.31 (0.05–1.76)	
ABCC5	TT	10	4	3	11	2	12	4	10	7	7
3933+313T>C	TC+CC	5	16	1	20	1	20	1	20	4	17
	p-Value		0.006		0.146		0.292		0.0648		0.049
	OR(CI)	0.13 (0.03–0.56)		0.18 (0.02–1.98)		0.30 (0.024–3.67)		0.13 (0.01–1.27)		0.24 (0.05–1.06)	
Xanthine oxidase	*AA*	6	9	0	8	0	8	1	7	1	7
1936A>G	*AG+GG*	9	11	4	23	3	24	4	23	10	17
	p-Value		0.258		0.335		0.447		0.432		0.161
	OR(CI)	1.23 (0.36–4.77)			ND		ND	1.22 (0.12–12.75)		4.12 (0.44–38.52)	
Xanthine oxidase	*AA*	6	9	1	14	1	14	2	13	3	12
2107A>G	*AG+GG*	9	11	3	17	2	18	3	17	8	12
	p-Value		0.258		0.326		0.435		0.368		0.137
	OR(CI)	1.23 (0.36–4.77)		2.47 (0.23–26.46)		1.56 (0.13–18.95)		1.15 (0.17–7.89)		2.67 (0.57–12.557)	

[a]Adverse reactions of anemia, leukopenia, and thrombocytopenia are included in myelosuppression. Analysis of Fisher's exact probabilities OR: odds ratio; p<0.05; 95%. CI, Confidence interval.

Classification of ADRs by NCI nomenclature

A total of 79 events of ADRs by MTX were scored according to NCI nomenclature. This classifies ADRs by severity, where grade 1 (G1), G2, and G3 correspond to a mild, moderate, and severe reaction, respectively (Table 5). Myelosuppression reaction (anemia, leukopenia, and thrombocytopenia) were the most frequent, with many patients who experienced G2 and G3 reactions characteristic of MTX. Analysis of the genetic polymorphisms regulating drug resistance proteins found that patients with the homozygote *ABCB1* variant *1236T>C* developed more

Table 5: Classification of adverse reactions according to the National Cancer Institute (NCI) nomenclature and the ATP-binding cassette transporter or xanthine oxidase genotype.

Polymorphism		Myelosuppression							Adverse reactions in other organs							
		Anemia		Leukopenia			Thrombocytopenia		Hepatotoxicity		Nephrotoxicity		Neurotoxicity		Mucositis	
		G1	G2	G1	G2	G3	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
ABCB1	*WT*	5	1	5	1	0	5	0	1	0	1	0	2	0	1	1
1236C>T	*HT*	3	0	3	0	0	6	1	1	1	1	0	3	0	2	4
	HM	5	4	5	4	0	2	3	1	0	1	0	1	0	4	1
ABCC5	*WT*	10	4	12	2	1	10	3	11	4	1	0	2	0	5	6
3414+434A>C	*HT*	2	0	1	1	0	2	0	1	0	1	0	3	0	1	0
	HM	1	1	4	1	0	1	1	1	1	1	0	1	0	1	0
ABCC5	*WT*	11	4	14	3	1	7	2	3	0	2	0	5	0	4	4
3933+313T>C	*HT*	1	0	1	1	0	2	0	0	1	0	0	0	0	0	0
	HM	1	1	2	0	0	4	2	0	0	1	0	1	0	3	2
XO	*WT*	2	1	4	0	0	1	1	0	0	0	0	1	0	1	0
1936A>G	*HT*	10	4	13	5	0	11	3	2	1	3	0	3	0	5	4
	HM	1	0	0	0	0	1	0	1	0	0	0	2	0	1	2
XO	*WT*	5	2	9	1	0	4	1	1	0	1	0	3	0	2	1
2107A>G	*HT*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HM	8	3	8	3	1	9	3	2	1	2	0	3	0	5	5

ABCB1 and ABCC5, ATP-binding cassette transporter gene subfamilies; XO, xanthine oxidase; WT, wild type; HT, heterozygote; HM, adverse Homozygote mutation; G1, Grade 1- mild reactions; G2, Grade 2- moderate adverse reactions; G3, Grade 3-severe.

events of myelosuppression (anemia, leucopenia, and thrombocytopenia).

For the two *ABCC5* SNPs studied, carriers with the wild-type genotype had a greater frequency of adverse hematologic reactions compared with those who were heterozygote or homozygous variants. In case of the *3414+434A>C* polymorphism, 14 (17%) events were anemia, 16 (20%) were leukopenia, and 13 (14%) were thrombocytopenia. ADRs in the majority of variant genotypes (n=5) were leukopenia. Similar findings occurred with the *3933+313T>C* genotype. The most frequent adverse reaction occurred in the WT subjects with 15 (19%) cases of anemia, 19 (24%) occurrences of leukopenia, and nine (11%) cases of thrombocytopenia. Subjects with HM carriers had more thrombocytopenic events.

Among *XO 1936A>G* polymorphic carriers, the heterozygotes developed similar numbers of myelosuppression reactions. In patients with the *2107A>G* polymorphism, the number of adverse reactions was higher among those with two allele variants -HM.

ADRs in other organs (hepatotoxicity, nephrotoxicity, neurotoxicity, mucositis) were less frequent. The XO polymorphisms 1936A>G, 2107A>G and ABCC5 3414+434A>C were not correlated with MTX adverse events. Some ADRs were present for a short time during treatment and were resolved upon correction or modification of the specific treatment plan. Oral mucositis was frequent in those receiving methotrexate. Eleven events occurred in the group WT with the two variant mutations both for ABCB1 1236C>T. Similar findings were seen in *3414+434A>C* ABCC5 homozygote mutations. Finally, carriers of XO polymorphisms presented the highest number of cases of oral mucositis.

Discussion

A total of 35 patients with the *ABCB1 1236C>T* genetic polymorphism were studied. The highest allelic proportion of this gene was 57%. This value is similar to that reported by Levran et al. [23]. The ABCB1 gene polymorphism results in the over-expression of transport proteins as a consequence of the chemotherapeutic agents, and might have resulted from the effects of treatment. A similar finding was reported by Colom et al. [24].

MDR1 proteins that are regulated by the *ABCB1* gene are associated with resistance to various drugs, such as digoxin and cyclosporine. This suggests that a variant TT genotype, instead of the CC wild-type genotype, is responsible for the high concentration of the noted drugs [25–26]. This may explain the MTX ADRs, including leukopenia, thrombocytopenia, and mucositis. The five adverse events observed were in patients with mutated *ABCB1 TT* variant

gene polymorphisms. Moreover, five adverse reactions were observed in patients with WT genotype. On canonical regression analysis, the variant variable was significantly different for anemia and leukopenia in groups with the variant allele versus WT. This finding is different from that reported by Kotnik et al. [27], who evaluated various polymorphisms similar to 3435C>T and 2677G>T/A but did not find an association with these adverse events.

The current study did not find severe neurotoxicity – one of the characteristics of MTX toxicity. This finding differs from that of Erddyi et al. [26]. Nevertheless, those authors reported that certain transporter carriers with wild-type and heterozygote genotypes show modest symptoms categorized as grade 1 neurotoxicity [28].

The allelic frequencies of these two variants has previously been reported in a Mexican population [29]. The results of the current study suggest that these genes are over-expressed via induction by the oncological agents in a way that is similar to MRP4. This concurs with the report of McAller et al. [30], who demonstrated the mechanisms of thioguanine, cadmium chloride, and antimonium tartrate as causal agents of the over-expression for MRP5 and MRP4 proteins. Mor-Cohen et al. [31] found that between 3% and 31.2% of the variant alleles in a Jewish population may explain the appearance of severe adverse reactions.

The ADRs seen in patients with the ABCC5 wild-type genotype were mild myelosuppression (G1), hepatotoxicity, and mucositis. However, a significant degree of neurotoxicity occurred in a heterozygote patient with a 3414+432A>C polymorphism. Five patients with the 3933+313T>C polymorphism developed mucositis, although this was less frequent than that reported among those with the WT genotype.

XO heterozygotes were for 1936A>G, which differs from findings in a Caucasian population with a genotypic frequency of 1 heterozygote among 19 patients [32]. No heterozygotes were found for the 2107A>G polymorphism; rather, homozygous variants predominated. This finding suggests that the genotypic frequency of the studied polymorphisms is variable in Mexican pediatric patients with ALL.

XO plays an important role in purine metabolism, and is the reason why such drugs as allopurinol and MTX that inhibit XO could alter the hepatic metabolism of hypoxanthine and xanthine to uric acid. This implies that there is an increase of these metabolites in plasma, which requires renal elimination. For this, the transitory increases of xanthine at the level of the kidney could result in renal damage. However, in this study, MTX nephropathy was not observed, which might be attributed to the low doses of MTX administered [33].

Conclusions

In our evaluated population, the XO polymorphisms 1936A>G, 2107A>G, and ABCC5 3414+434A>C were not correlated with MTX adverse events. SNPs 1236C>T of ABCB1 and ABCC5 3933+313T>C were not associated with development of ADRs by MTX, but rather showed a probable protective effect for myelosuppression in patients with ALL. This is a fact that should be taken into consideration for the management of treatment with MTX in patients with ALL.

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