Original Article

Direct-acting Antiviral Agents Resistance-associated Polymorphisms in Chinese Treatment-naïve Patients Infected with Genotype 1b Hepatitis C Virus

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Abstract

Background: It has been reported that several baseline polymorphisms of direct-acting antivirals (DAAs) agents resistance-associated variants (RAVs) would affect the treatment outcomes of patients chronically infected with hepatitis C virus (CHC). The aim of this study is to investigate the prevalence of DAAs RAVs in treatment-naïve GT1b CHC patients.

Methods: Direct sequencing and ultra-deep sequencing of the HCV NS3, NS5A, and NS5B gene were performed in baseline serum samples of treatment-naïve patients infected with genotype 1b hepatitis C virus (HCVs).

Results: One hundred and sixty CHC patients were studied. Complete sequence information was obtained for 145 patients (NS3), 148 patients (NS5A), and 137 patients (NS5B). Treatment-failure associated variants of DAAs were detected: 56.6% (82/145) of the patients presented S122G for simeprevir (NS3 protease inhibitor); 10.1% (14/148) of the patients presented Y93H for daclatasvir and ledipasvir (NS5A protein inhibitors); 94.2% (129/137) of the patients presented C316N for sofosbuvir (NS5B polymerase inhibitor). Nearly, all of the DAAs RAVs detected by ultra-deep sequencing could be detected by direct sequencing.

Conclusions: The majority of genotype 1b CHC patients in China present a virus population carrying HCV DAAs RAVs. Pretreatment sequencing of HCV genome might need to be performed when patients infected with GT1b HCV receiving DAAs-containing regimens in China. Population sequencing would be quite quantified for the work.

Key words: Antiviral Resistance; Direct Antiviral Agents; Hepatitis C Virus

Introduction

Hepatitis C virus (HCV) is one of the main causes of liver cirrhosis and hepatocellular carcinoma, where approximately 350,000 deaths occur each year due to HCV-related liver diseases. The latest estimation indicates that more than 185 million people around the world (2.8% of the world population) have been infected with HCV.[1] In China, approximately 2.2% of the population on average were infected with HCV (most of which were genotype 1b), with a distribution range from 2.1% in Fujian province to 9.6% in Henan province.[2]

To inhibit the global prevalence of HCV infection, various strategies with regard to therapeutic medication have been developed. The traditional standard of care contains pegylated interferon plus ribavirin (RBV), which could help genotype 1b patients to achieve sustained virological response (SVR) rates of 45%, further up to 65–75% for genotype 2/3 patients.[3] Although patients chronically infected with hepatitis C virus (CHC) in Asia carries more beneficial IL28B genotypes, nearly 20% of them could not achieve SVR.[4] In this regard, more than 30 newly developed direct-acting antivirals (DAAs) have readily been approved in the Europe, North American, and Japan or are currently being in phase 2/3 clinical trials. DAAs

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mainly encompass NS3/4A protease inhibitors (PIs, such as telaprevir, boceprevir, simeprevir, MK-8742, asunaprevir, etc.), NS5A inhibitors (such as daclatasvir, ledipasvir [LDV], MK-5172, etc.), and NS5B polymerase inhibitors (such as sofosbuvir [SOF], dasabuvir, etc.). In general, they are effective toward pan-genotypic HCV by helping patients to achieve high SVR rates up to 100%, especially genotype 1b CHC patients.[5]

The high replication rate of HCV, along with the low fidelity and poor proof-reading of its polymerase, generates a highly variable virus population denoted as "quasispecies." The creation of variants encoding amino acid substitutions may result in reduced susceptibility to antiviral agents.[6] Compared with HIV, the rate of emergence of resistant variants is much higher for HCV. It is estimated that the error rate of the HCV polymerase is 10-fold higher than that of HIV, and the rate of production of RNA virus is 100-fold higher than that of HIV.[7] Data from clinical trials have revealed that baseline resistance-associated variants (RAVs) were correlated with treatment outcomes of DAAs. For example, patients presented PIs RAVs, such as Q80K, D168E/V, NS5A RAVs L31M/V, Y93H, and NS5B polymerase inhibitors RAVs L159F, C316N, V321A achieved SVR rates much lower than those who did not.[8-10]

RAVs could exist even as minor variants at baseline, which would rapidly become the main strain under selective pressure, subsequently leading to a treatment-failure. Ultra-deep sequencing could detect minor variants with mutation frequencies of <1%. However, compared with population sequencing, the expense for ultra-deep sequencing was relatively high. Since none of the DAAs has been approved in China, there have been seldom reports by far on baseline DAAs RAVs in our country. In turn, it remains unclear if it is necessary to detect baseline DAAs RAVs before DAAs treatment. In this study, we perform both population sequencing and deep sequencing to detect baseline DAAs RAVs in genotype 1b CHC patients in China.

Methods

Study design

Serum samples were derived from 160 treatment-naïve patients chronically infected with genotype 1b HCV. Blood samples were collected and stored at −20°C.

Population sequencing was carried out in 160 patients to figure out the prevalence of RAVs in GT1b treatment-naïve CHC patients. Ultra-deep sequencing was carried out in 23 patients to find out minor variants with low mutation frequencies. Written informed consent was obtained from all the patients. The study was conducted in accordance with the International Society for Pharmacoepidemiology Guidelines for Good Epidemiology Practices and applicable regulatory requirements. HCV viral load (Abbott Real Time HCV; Abbott Laboratories, Des Plaines, IL, USA), HCV genotype (Versant HCV Genotype 2.0 [LiPA]; Siemens Healthcare Diagnostics, Tarrytown, NY, USA), and IL28B genotype (iPLEX Gold; Sequenom, San Diego, CA, USA), were conducted.

RNA extraction, polymerase chain reaction, and sequencing

HCV RNA was extracted from 140 μl ethylenediaminetetraacetic acid anticoagulated plasma using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) after centrifugation at 24,000 × *g* for 1 h at 4°C. The extracted RNA was transcribed to cDNA and the NS3, NS5A, and NS5B fragments were amplified by polymerase chain reaction (PCR) in a one-step process (Superscript III One-step RT-PCR with platinum Taq kit; Invitrogen, Carlsbad, CA, USA) following the manufacturers' instructions. The primers used are listed in Table 1. Cycling conditions included an initial cDNA synthesis step at 55°C for 30 min, followed by a denaturation step at 94°C for 2 min, 40 cycles of PCR amplification (94°C for 15 s, 58°C for 30 s, 68°C for 2 min), and a final 10 min extension step at 68°C. The PCR mix contained 25 μL of 2× reaction mix, 1 μL of each primer, 8 μL of extracted RNA as template, and nuclease-free H_2O to a final volume of 50 μL. The PCR reaction was carried out with Thermal cycler PCR machine (Thermo, CA, USA). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). In the deep sequencing study, the amplified NS3, NS5A, and NS5B fragments were modified by the Multiplexing Sample Preparation Kit (Illumina, San Diego, CA, USA), and sequence analysis was performed by Illumina Hiseq 2000. In the population study, the amplified fragments were subjected to direct sequencing by ABI 3730xl DNA sequencer (ABI, USA).

Sequence alignment and analysis

The BioEdit 7.09 software (Borland company, USA) was used for editing the sequences. Translation from the nucleic acids sequences into amino acids sequences was performed with RevTrans 2.0 (Technical university of Denmark, Denmark). Sequences were compared with the reference sequence of

Table 1: Primers used for amplifying the NS3, NS5A and NS5B regions

Gene fragments	Primers	5'-3'sequence
NS3	Forward 1	5'-GCCGCGATGCCATCATCC-3'
	Reverse 1	5'-CATTAGAGCGTCTGTTGC-3'
	Forward 2	5'-CTATGGCAAAGCCATCCC-3'
	Reverse 2	5'-GCCAGACTCCCTTGTACCC-3'
NS5A	Forward 1	5'-GTGGAAGTGTCTCAYACG-3'
	Reverse 1	5'-ATGTTYCCGCCCATCTCCTGCCG-3'
	Forward 2	5'-CCCCACGCACTATGTGCC-3'
	Reverse 2	5'-TARAGGGCCATYTTCTCGC-3'
NS5B	Forward 1	5'-TCACAGCTCCCATGYGAGCC-3'
	Reverse 1	5'-CTTYGCAGCTCGACAGGC-3'
	Forward 2	5'-ACCGGGACGTGCTBAAGG-3'
	Reverse 2	5'-GGGGAGCAGGTAGATGCC-3'
	Forward 3	5'-CGCTGYTTTGACTCAACGG-3'
	Reverse 3	5'-ATTGGCCTGGAGTGTTTAGC-3'

HCV-1b (AJ238799). The sequence alignment was performed with Clustal X 2.0 (University College Dublin, Irland). The reported amino acid substitutions associated resistance to DAAs according to previously reported data were scored: V36A/M, F43S, T54S, Q80K, S122G, R155K, D168E/V, V170I, etc., for NS3/4A PIs; and L23F, L28T, R30Q, L31M/V, P32L, Q54H, P58S, Q62H, Y93H, etc., for NS5A protein inhibitors; T19S, N142T, S282T, A442T, S556G, etc., for NS5B RAVs.

Statistical analysis

SPSS 21.0 (IBM, USA) was employed to determine the statistical differences. Continuous data were presented as the mean ± standard deviation (SD). Nonparameters Mann–Whitney *U*-test, Chi-square test with Yates correction, or Fisher exact test was performed. $P < 0.05$ was considered as statistically significant.

Results

Patient characteristics

In total, 160 treatment-naïve patients CHC genotype 1b were enrolled in this study. Fifty-two percent of patients were male, and the median age at testing was 46.5 (range, 27–65) years [Table 2].

Table 2: Baseline characteristics of the study population

Items	Values
Age (year), median (range)	46.5 (27–65)
Male, *n* (%)	72 (52.6)
BMI (kg/m^2), mean ± SD	22.7 ± 0.2
ALT (U/L), median (range)	36 (16–277)
AST (U/L), median (range)	43.5 (18–292)
PLT count (×10^9/L), median (range)	188 (92–379)
AST/PLT, median (range)	0.24 (0.09–1.40)
Hemoglobin (g/dl), median (range)	138.5 (110–178)
IL28B genotype (*n*)	
CC	103
CT	34
Baseline HCV RNA >800,000 (IU/ml), *n* (%)	138 (86.3)

BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelet; HCV: Hepatitis C virus.

Over 50% of patients presented PI resistance-associated variants NS3-S122G

One hundred and forty-five patients (90.6%) were successfully amplified with the NS3 fragments, 71% (103/145) of whom presented at least one PIs RAVs. About 56.6% (82/145) of the patients presented S122G variant, 33.1% (48/145) of the patients presented V132I variant, 13.1% (19/145) of the patients presented V170I variant, and 5.5% (8/145) of the patients presented T54S variant [Table 3]. Nucleoside changes at codon position 36, 55, and 80 were detected but could cause only synonymous substitutions.

As results by ultra-deep sequencing, the average mutation frequencies were 23.6% for S122G variants, 75.4% for V132I and 33.8% for V170I. All of the RAVs detected by ultra-deep sequencing could be detected by population sequencing.

Table 3: Number of patients harboring NS3/4A PIs and NS5A protein inhibitors RAVs

DAAs	RAVs	Number of patients (%)	Drugs
NS3/4A PIs	T54S	8/145 (5.5)	Telaprevir, boceprevir, simeprevir, and faldaprevir
	S122G	82/145 (56.6)	Telaprevir, simeprevir
	V132I	48/145 (33.1)	Only *in vitro*
	V170I	19/145 (13.1)	Telaprevir
NS5A protein inhibitors	R30Q	9/148 (6.1)	Daclatasvir, ledipavir, and samatasvir
	Q54H	13/148 (8.8)	Daclatasvir, ABT-267
	P58S	3/148 (2.0)	Daclatasvir
	Q62H	5/148 (3.4)	Daclatasvir
	A92T	3/148 (2.0)	Only *in vivo*
	Y93H	14/148 (10.1)	Nearly all NS5A protein inhibitors

DAAs: Direct-acting antivirals; RAVs: Resistance-associated variants; PI: Protease inhibitor.

NS5A PI resistance-associated variants Y93H were found, and L31M variants were detected only by deep sequencing

About 92.5% (148/160) of the patients were successfully amplified with the NS5A fragments, 14.9% (22/148) of whom presented at least one NS5A protein inhibitors RAVs. About 6.1% (9/148) of the patients presented Q30R variant, 8.8% (13/148) of the patients presented Q54H variant, 10.1% (14/148) of the patients presented Y93H variant. Only 3 patients presented P58S variant, and only 5 patients presented Q62H variant. Only synonymous substitutions were detected at amino acid sites 23, 28, and 32. Nucleoside substitutions from A to G at 6347 site were detected in 42.6% (63/148) patients, which could only cause synonymous substitutions at amino acid site 31 [Table 3].

According to the results of ultra-deep sequencing, the average mutation frequencies were 61.0% for Q30R, 13.5% for Q54H, 95.7% for Q62H, 14% for Y93H. Interestingly, the daclatasvir, LDV, and MK-8742 RAVs L31M have not been detected by population sequencing in any one of the 148 patients but have been detected by ultra-deep sequencing in 3 patients, with average mutation frequencies of 4.7%.

High percentage of patients presented NS5B polymerase inhibitors resistance-associated variants C316N

The fragments of NS5B were successfully amplified in 85.6% (137/160) patients. Nearly, all patients presented at least one RAV to polymerase inhibitors at baseline, except for one.

When considering RAVs of nucleoside inhibitors, 94.2% (129/137) of the patients were detected harboring the C316N variant. When IL28B genotype was regarded, the prevalence of C316N variant between patients who carried CC and non-CC genotype was identical. There were also no significant differences between the patients with and without C316N variant for clinical characteristics, except for the hemoglobin levels ($P = 0.004$). Most of the patients

harbored A338V (83.3%). However, L159F variant, which was always detected with C316N variant simultaneously, has not been detected in any of patients in our study. At the codon position 282 of NS5B, the primary SOF resistant associated variant, only synonymous variant (AGC<->AGT) was detected. Other important RAVs to NS5B polymerase inhibitors, such as L320F, V321A, and V499A, were detected in our study. In addition, only three patients presented S142 at baseline, whereas none of the patients exhibited the resistant type N142T [Tables 4 and 5].

It is worth-mentioning that some RAVs of nonnucleoside inhibitors were also detected in our study, such as V494A/T/L, V499A (thumb I); M423I/T, M424V, M426T (thumb II); H95Q, M414I/L, S556G/N (palm I); S365A/L/S/T (palm II), etc., Regarding the cross-resistance between these four categories, we have worked out the possible overlap of RAVs of different sorts of nonnucleoside inhibitors. Most of the patients presented RAVs belonging to certain one category, for instance, the most epidemic RAV C316N pertained to palm II. Only one patient exhibited RAVs of four categories. None of the patients harbored RAVs of active sites [Table 4].

As results by ultra-deep sequencing, the average mutation frequencies were 41.7% for C316N, 24.8% for A338V, 26.5% for A442T, and 31.4% for S556G. Interestingly, L419M variant, which was reported to be resistant to VCH-759 and VCH-916, had not been detected by population sequencing and were detected in two patients as minor variants (with average mutation frequencies of 1.6%).

Table 4: Number of patients harboring NS5B polymerase inhibitors RAVs (n = 137)

Domain	RAVs	n (%)
Active site	N142T	6/137 (4.4)
Palm I	T19S	49/137 (35.8)
Palm I	M71V	2/137 (1.5)
Palm I	H95Q	6/137 (4.4)
Palm I	M414I/L	6/137 (4.4)
Palm I	S556G/N	5/137 (3.7)
Palm II	C316N	129/137 (9.4)
Palm II	A338V	109/137 (8.0)
Palm II	S365A/L/S/T	1/137 (0.7)
Thumb I	V499A	23/137 (16.8)
Thumb I/II	V494A/T/L	14/137 (10.2)
Thumb II	M423I/T	3/137 (2.2)
Thumb II	M424V	24/137 (17.5)
Thumb II	M426T	2/137 (1.5)
Thumb II	L392I	1/137 (0.7)
Thumb II	A442T	22/137 (16.1)

RAVs: Resistance-associated variants.

Discussion

In our study, as many as 71% of genotype 1b treatment-naïve patients presented at least one NS3/4A PIs RAV. The most prevalent variant was S122G (56.6%), which has been confirmed as one of the major RAVs of simeprevir (one of the first-generation second-wave PIs).[11] In CONCERTO trial conducted in Japan, the baseline prevalence of S122G variant was relatively lower (34.9%) than that of our findings, while the baseline prevalence of V170I variant was relatively higher (39.6% vs. 13.1%) than our findings.[12,13] T54S variant detected in our study was also correlated with resistance to simeprevir, with relatively low prevalence (5.5%). Mutations associated with first-generation first-wave PIs detected in this study were T54S (telaprevir and boceprevir), S122G (telaprevir), and V170I (telaprevir).[14] However, these RAVs only conferred low-level resistance to these two licensed DAAs. With respect to other reports,[15-17] none of other baseline polymorphisms correlated with treatment outcomes of PIs were detected in our study. For example, R155K and D168E/V variants were RAVs of asunaprevir (had licensed

Table 5: Baseline characteristics of the patients with and without C316N variant

Baseline characteristics	Wild type (n = 8)	Mutation type (n = 129)	P
Age (year), median (range)	45 (38–54)	50 (23–67)	0.600
Male, n (%)	55 (50)	4 (100)	0.150
BMI (kg/m^2), average (range)	22.3 (22.0–30.6)	26.4 (16.9–32.2)	0.780
ALT (U/L), median (range)	66 (63–155)	223.5 (16–404)	0.070
AST (U/L), median (range)	34 (33–192)	137 (18–292)	0.380
PLT count (×10^9/L), mean ± SD	202 ± 60	214 ± 30	0.790
AST/PLT, mean ± SD	0.18 ± 0.05	0.59 ± 0.40	0.470
Hemoglobin (g/dl), median (range)	171.5 (163–175)	144.5 (95–177)	0.004
Response to Peg-IFN plus RBV			
SVR (n = 77)	2	75	0.040
Treatment-failures (n = 60)	7	53	
IL28B genotype			
CC (n = 103)	5	98	0.300
CT (n = 34)	4	30	
Baseline HCV RNA >800,000 (IU/ml), n (%)	6 (75)	112 (86.8)	0.700

BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelet; Peg-IFN: Pegylated interferon; RBV: Ribavirin; SVR: Sustained virological response; HCV: Hepatitis C virus.

in combination with daclatasvir as interferon [IFN]-free regimens in Japan) and MK-5172 (one of the second generation PIs),[18,19] none of which were found. This discrepancy might be due to the fact that virus of different quasispecies even within the same subtype has different mutations.[17] Since all of the RAVs detected by ultra-deep sequencing could be detected by population sequencing, we can conclude that population sequencing could qualified for baseline PIs RAVs screening in China.

As one of the most important RAVs of most of the NS5A protein inhibitors,[20] Y93H variants were detected in 10.1% of patients included in our study. However, L31M variants, another mutation associated with treatment outcomes of LDV, daclatasvir, and ABT-267,[10,21,22] were only detected by ultra-deep sequencing in three patients with low mutation frequencies. Several *in vitro* studies have reported that single L31M variant could only lead to 3-fold change with EC50 of daclatasvir. Single Y93H variant could lead to 24-fold change of it while combination mutations of L31M plus Y93H could lead to 7105-fold change with EC50 of daclatasvir.[23-25] In addition, single Y93H variant could lead to 77-fold change of EC50 of ABT-267 while L31M plus Y93H could lead to 142-fold change of it.[26] What's more, Y93H variant could lead to extremely high-level (994-fold) fold-change with EC50 of LDV. L31M-Y93H joined mutations occurred only in one patient in our study. Although NS5A protein inhibitors were usually used in combination with PIs (such as asunaprevir), baseline polymorphisms of L31M or Y93H could make patients to experience virologic relapse more easily and achieved much lower SVR rates.[27] Data by ultra-deep sequencing revealed that most of the NS5A protein inhibitors RAVs detected here were with relatively high mutation frequencies, except for L31M variants. Nevertheless, L31M variants occurred only in three patients with low mutation frequencies. In this way, population sequencing might be qualified for baseline NS5A protein inhibitors RAVs screening in China.

The detection of naturally occurring RAVs of NS5B polymerase inhibitors at baseline has been reported in numerous studies. Some of them may influence the treatment outcomes of DAAs-containing regimens. In the clinical trials of SOF-containing regimens, the primary concern lies in the S282T, an important variant associated with treatment-failure.[28] However, a representative study conducted by FDA from the US suggested that NS5B-C316N was found to be associated with treatment failure when patients CHC genotype 1b receiving SOF plus RBV therapy, throughout re-systemizing all the data from SOF-containing trials where ultra-deep sequencing was performed.[9] In the clinical trial P7977-2025, 16/61 posttransplantation patients failed therapy of SOF plus RBV, where 6/16 patients harbored C316N at baseline, in other words, all of the six patients did not achieve SVR. On the contrary, for the other 45 patients who achieved SVR12, no detection of C316N was reported. Eighty-two percent (45/55) of the patients who did not carry C316N pretreatment achieved SVR12. However, 66.7% (4/6) of patients harbored L159F at the same time.[29] In clinical trial NEUTRINO, 4/66 of the patients harbored C316N variant at baseline, 50% of which (2/4) experienced virologic relapse after receiving treatment of SOF plus RBV. In this trial, 85% of the patients who did not harbor this mutation achieved SVR12.[30]

RAV C316N is located in palm II (allosteric). Despite the fact that the majority of RAVs of SOF reported are at the active sites (such as S282T and N142T), position 316 was deemed as the only different residue within 8 A in the NS5B polymerase between HCV genotype 1a and 1b. N316 in HCV GT1b polymerase structure is much larger than C316, which might lead to the block of the active site of SOF. However, C316 has been reported to be relatively conserved in GT1a, but polymorphic in GT1b.[9] The prevalence of C316N variant was quite different in different countries. Data from Los Alamos databank showed that the prevalence of C316N was much higher in Asia than worldwide (91.6% vs. 36%) as well as American continent (from 0% to 18%).[31] In our study, both the ultra-deep sequencing and population sequencing revealed that the prevalence of C316N variant in treatment-naïve Chinese GT1b CHC patients was quite high, which is in good agreement with previous findings.[31,32]

As part of IFN-free regimens, SOF in combination with LDV with and without RBV has been one of the most important regimens nowadays.[33] Combination treatment could help patients to overcome antiviral resistance more easily. However, cross-resistance was a new challenge in this circumstance. In our study, data showed that the patient presented NS5A-L31M-Y93H joined variants as previously stated, simultaneously harbored NS5B-C316N. Even in the combination regimens of SOF plus LDV, baseline polymorphisms of Y93H were correlated with virologic failure. And results by ultra-deep sequencing showed that the mutation frequencies increased dramatically at the time of relapse.[22]

Both RAV S282T and N142T were at the active site of NS5B polymerase, but none of the 137 patients exhibited the existence of these two variants. This might be due to the reason that the active site was conserved.[34] Our findings are consistent with the data from the Los Alamos databank. Since most of the patients presented RAVs of one category, cross-resistance seems less likely to occur. Data by ultra-deep sequencing revealed that most of the NS5B polymerase inhibitors RAVs detected here were with high mutation frequencies, especially for C316N variants. Nevertheless, L419M variants occurred only in two patients with low mutation frequencies. In this way, population sequencing might qualify for baseline NS5B polymerase inhibitors RAVs screening in China.

In conclusion, we have employed both the ultra-deep sequencing and population sequencing methods to detect DAAs RAVs in genotype 1b treatment-naïve patients, among which the prevalence of variants related with

treatment-failure of NS3/4A PIs (S122G), NS5B polymerase inhibitors (C316N) and NS5A inhibitors (Y93H) were quite high, in particular, C316N. Currently high prevalence and high mutation frequencies of RAV C316N in China might impede the virologic response when distributing SOF-containing regimens in the near future. Our findings suggest that pretreatment sequencing of HCV genome might need to be performed when patients infected with GT1b HCV receiving DAAs-containing regimens in China. Population sequencing would be quite suitable for the work.

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Conflicts of interest

There are no conflicts of interest.

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