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Potential Increase in the Prognostic Value of *p53* Mutation by *Pro*72 Allele in Stage I Non-Small-Cell Lung Cancer

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ABSTRACT

Background. Accumulated evidence suggests that p53 function altered by its gene mutation or genetic polymorphism contributes to tumor malignancy. Association of *p53* mutation and its codon 72 polymorphism with lung cancer prognosis has been extensively studied. However, the joint effect of *p53* mutation and *p53* codon 72 polymorphism on lung cancer prognosis remains uncertain.

Methods. In the present study, 266 primary lung cancer patients were included and overall survival was calculated. Genomic DNA prepared from adjacent normal lung and lung tumor tissues was used to determine *p53* codon 72 genotype and *p53* mutation by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) and direct sequencing, respectively.

Results. For all stages, neither *p53* codon 72 genotype nor *p53* mutation is associated with lung cancer prognosis. However, stage I patients with *p53* mutation had a 1.79-fold hazard ratio [95% confidence interval (CI) 1.04–3.10] for overall survival when compared with *p53* wild-type patients. Notably, stage I patients with *p53* mutation and *p53* codon 72 *Pro*/*Pro* genotype experienced a 2.66-fold hazard ratio (95% CI 1.21–5.85) for overall survival when compared with those with *p53* wild-type and *Arg*/*Arg* genotype. An increased prognostic value was not observed in stage I patients with *p53* wild-type and *p53 Pro*72 allele or in those with *p53* mutation and *p53* codon 72 *Arg*/*Arg* genotype.

Conclusions. We therefore suggest that *p53* codon 72 *Pro* allele potentially increases the prognostic value of *p53* mutation in stage I non-small-cell lung cancer.

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Mutations in *p53* occur in at least 50% of all cancers, and over 90% of these mutations eliminate the ability of the p53 protein to bind to its DNA targets.[1] Further, *p53* tumor suppressor functions have been used to examine the behavior of *p53* mutant tumor cells or correlated loss of specific p53-controlled functions on tumor progression in animal models.[2,3] For example, loss of *p53* dramatically accelerated tumor development and induced a phenotypic switch in transgenic mice with lung-targeted expression of CRaf kinase in a study on gene alteration frequency in human lung adenocarcinoma and its effect on tumor progression.[2] In addition, the influence of *p53* loss on malignant progression was observed as early as 6 weeks after tumor initiation in knock-in mice with mutations in K-*RAS* combined with a null or mutant allele of *p53* gene.[3] However, another report showed lack of prognostic significance of *p53* mutations in primary resected non-small-cell lung cancer (NSCLC).[4] Other studies have indicated that *p53* mutation is associated with the clinical outcome in stage I lung cancer, but not in late-stage lung cancer.[5,6] Thus, the role of *p53* mutation as a prognostic factor in lung cancer is still controversial.

A single-nucleotide polymorphism in the *p53* gene resulting in the substitution of arginine (*Arg*) by proline (*Pro*) at codon 72 has been identified and shown to be

important for the apoptotic functions of p53 protein.[7] The *Arg*72 variant induces apoptosis and represses cellular transformation markedly better than the *Pro*72 variant. The *Pro*72 variant, in turn, induces a higher level of G1 arrest than *Arg*72 variant.[7] It has also been shown that the *Pro*72 variant induces transcription of DNA repair genes, and *Pro*72-expressing cells have higher DNA repair capability than corresponding *Arg*72 variant.[8] The association of *p53* Arg72Pro polymorphism with lung cancer susceptibility has been extensively studied, but with inconsistent results.[9–12] Most studies have indicated that Arg72Pro polymorphism is not associated with lung cancer prognosis.[13–15] However, one study found that patients with the *Pro*/*Pro* genotype tend to have poorer prognosis than those with the *Arg*/*Pro* genotype.[16]

Another report indicated that the presence of *Arg*72 allele is associated with significantly higher frequency of *p53* mutation in oral squamous cell carcinoma patients.[17] Langerød et al.[18] investigated correlations between the codon 72 polymorphism and somatic *p53* mutations in breast cancer and colon cancer cells and noted that *p53* mutations are commonly seen on the *Arg*72 allele in breast carcinomas but not in colon carcinomas. In contrast, lung tumors with *Pro*72 allele have higher frequency of *p53* mutation than those with *Arg*72 allele.[14] We thus hypothesized that the combined effects of somatic *p53* mutation and *Pro*72 allele are more contributive to lung cancer prognosis than *p53* mutation or *p53* codon 72 polymorphism alone.

PATIENTS AND METHODS

Patients

The study protocol conformed to Declaration of Helsinki guidelines and was approved by the participating institutions. From 1993 to 2005, surgical specimens were obtained from 266 patients with primary NSCLC at Taichung Veterans General Hospital, Taichung, Taiwan. Tumor types and stages were determined according to World Health Organization (WHO) classification by board-certified pathologists. Complete follow-up was conducted for all subjects. The end of the follow-up period was June 2005. None of the subjects had received neoadjuvant chemotherapy or radiotherapy before surgery. Relapse time was determined from date of surgery to date of local recurrence or systemic metastasis in 116 patients. Overall survival was calculated from date of surgery to date of death or last follow-up. Median follow-up time was 26.5 months (range 1.1–135.4 months). There were 181 deaths. Demographic data for each individual including age, gender, and smoking habits were collected from patient interviews and review of hospital charts with informed consent. In terms of smoking status, patients were classified as smokers if they were active or previous smokers or as nonsmokers if they had never smoked.

Genomic DNA Extraction

Tissue samples from patients were isolated from surgically resected lung tumors and normal tissues adjacent to lung tumors. These tissues were immediately snap-frozen and subsequently stored at –80°C. To obtain genomic DNA, tissue specimens were stained with hematoxylin and eosin. Then genomic DNA was isolated from the tissue samples using proteinase K digestion and phenol–chloroform extraction, followed by ethanol precipitation.

Genotyping of p53 Codon 72 Polymorphism

Genotyping analysis of the *p53* codon 72 polymorphism from genomic DNA samples of adjacent normal lung tissue was conducted as described previously.[19] For PCR-RFLP analysis, PCR amplification primers were 5′-ATCTACAG TCCCCCTTGCCG-3′ (forward) and 5′-GCAACTGAC CGTGCAAGTCA-3′ (reverse). A 296-base pair (bp) fragment was amplified using a PCR program starting with denaturation for 5 min at 95°C, followed by 35 cycles of 40 s at 95°C, 40 s at 60°C, and 40 s at 72°C. The amplifying fragment was digested by *Bst*UI (recognition site CGCG; New England Biolabs, Ipswich, MA, USA). Digestion products were analyzed by electrophoresis through a 2% agarose gel stained with ethidium bromide. The *Arg* allele contained a *Bst*UI restriction site, thus homozygous *Arg*/*Arg* individuals had two fragments of 169 and 127 bp. Homozygous *Pro*/*Pro* individuals had a single fragment of 296 bp, and heterozygous *Arg*/*Pro* individuals revealed all three fragments. All genotype reading was done blind to disease status of study subjects. Rigorous quality control procedures were applied throughout the genotyping process. To avoid PCR contamination, reagents for PCR reaction were carefully aliquoted, and each aliquot was used no more than three times. For each assay, a negative control (no DNA template) was added to monitor PCR contamination. Pilot experiments were conducted to optimize the restriction digestion conditions. After genotyping of each genetic polymorphism, ~20% of the samples were randomly selected for repeat assays to validate the results.

Sequencing of p53 Gene

Direct sequencing of PCR products amplified from lung tumor tissues isolated from all patients was performed to

identify mutations in exons 5–8 of the *p53* gene as described previously.[20] The DNA of tumor cells was isolated by microdissection of the lung tumor tissues. DNA lysis buffer was used to lyse cells and the solution was subjected to proteinase K digestion and phenol–chloroform extraction. Finally, the DNA was precipitated by ethanol with the addition of linear polyacrylamide to increase the efficiency of DNA precipitation. Target sequences were amplified in a 50 µl reaction mixture containing 20 pmol of each primer, 2.5 units Taq polymerase (TAKARA Shuzo, Shiga, Japan), 0.5 mM deoxyribonucleotide triphosphates (dNTPs), 5 µl PCR reaction buffer, and 1 µl genomic DNA as the template. Genomic DNAs extracted from the frozen sections were not adequate for amplification of long fragment DNAs, and therefore PCR products ranging from 200 to 400 bps were amplified for *p53* mutation analysis. Primers for β-actin, acting as an internal control, were included in each amplification reaction. The primers used in the reactions were E5S (5′-TGCCCTGA CTTTCAACTCTG-3′) and E5AS (5′-GCTGCT CAC-CATCGCTATC-3′) for exon 5, E6S (5′-CTGATTCCTC ACTGATTGCT-3′) and E6AS (5′-AGTTGCAAACCAGA CCTCAGG-3′) for exon 6, E7S (5′-CCTGTGTTATCT CCTAGGT TG-3′) and E7AS (5′-GCACAGCAGGCCA GTGTG CA-3′) for exon 7, and E8S (5′-GACCT GAT-TTCCTTACTGCC-3′) and E8AS (5′-TCTCCT CCAC CGCTTCTTGT-3′) for exon 8. An initial cycle was performed for 5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 54°C, and 1 min at 72°C. The PCR products were sequenced using an Applied Biosystems 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the same primers used for PCR were used for DNA sequencing. All *p53* mutations were confirmed by direct sequencing of both DNA strands.

Statistical Analysis

Student's *t* test and the χ^2 test were applied to continuous or discrete data analysis. The associations among the *p53* codon 72 genotypes, *p53* mutation status and patient survival were determined using the Kaplan–Meier method and assessed using the log-rank test. Multivariate analysis was performed by applying a Cox proportional hazard model to calculate the hazard ratio and its 95% confidence interval for age, gender, smoking status, tumor type, T value, *p53* genotypes and *p53* mutation status. All statistical analyses were conducted using the SPSS statistical software program, version 11.0 (SPSS, Inc., Chicago, IL, USA) and all statistical tests were two-sided. P values < 0.05 were considered statistically significant.

RESULTS

Pro72 Allele is Common in Late-Stage Tumors and p53 Mutation

A previous study indicated that *Pro*72 variant is associated with a marked reduction in apoptosis when compared with *Arg*72 variant.[7] In the present study, we thus combined *Arg*/*Pro* and *Pro*/*Pro* genotypes to acquire sufficient statistical discriminatory power to explore the role of *p53* codon 72 genotypes in NSCLC. Our data showed that *Pro72* allele (*Arg*/*Pro* + *Pro*/*Pro* genotypes) was more common in stage II + III tumors than in stage I tumors ($P = 0.04$, χ^2 test, Table 1). In addition, male patients with *Pro* allele (72.0%) were more prevalent than female patients with *Pro* allele (60.8%, $P = 0.07$). However, this polymorphism was not associated with other clinical parameters including age, smoking status, tumor type, or T and N values (Table 1). Interestingly, *p53* mutation was more frequently observed in tumors with *Pro72* allele than in tumors with *Arg*/*Arg* homozygous genotype ($P = 0.04$). Subsequently, we analyzed the association of *p53* mutation pattern in tumors and *p53* codon 72 polymorphism (Table 2). Among all types of mutations, G:C to T:A (19.4%), deletion/insertion mutation (19.4%), A:T to G:C (19.4%), and G:C to A:T (40.9%) were predominant. However, no significant difference was observed in the *p53* mutation patterns for the *p53* codon 72 polymorphism.

p53 Mutation May Act as an Independent Prognostic Indicator in Stage I Lung Cancer and *Pro72* Allele Increases the Prognostic Value of *p53* Mutation.

To elucidate whether *p53* mutation or *Pro72* allele is associated with lung cancer prognosis, univariate analysis was performed to obtain the hazard ratios (HR) for overall survival (OS) for *p53* mutation status, *p53* codon 72 genotypes, and tumor clinical parameters. Patients with male gender (HR = 1.87; 95% CI 1.32–2.65), T3 + T4 (HR = 1.61; 95% CI 1.16–2.25), N1 + N2 + N3 (HR = 1.94; 95% CI 1.44–2.61), and stage II + III (HR = 1.83; 95% CI 1.34–2.50) had higher HR for OS than those with female gender, T1 + T2, N0, and stage I, respectively (Table 3). However, no statistical association was found for other parameters, including age, smoking status or tumor type. Interestingly, when stratified by tumor stage, stage I patients with *p53* mutation had a 1.79-fold HR (95% CI 1.04–3.10) for OS when compared with *p53* wild type (Table 4). However, such relationship was not observed among stage II + III patients. In addition, *p53* codon 72 genotype was not associated with prognosis, even under stratification by tumor stage. Further, the prognostic value of *p53* mutation combined with *p53* codon 72 genotype was analyzed by Cox regression model (Table 5). When stage I lung cancer

TABLE 1 Relationships among clinical parameters and p53 codon 72 genotypes, p53 mutation status in 266 NSCLC patients

Variables	No. of patients	*p53* codon 72 polymorphism	
		Arg/Arg (%)	*Arg/Pro* + *Pro/Pro* (%)
Total	266	83 (31.2)	183 (68.8)[a]
Age (years, mean ± SD)		65.0 ± 10.2	64.1 ± 9.7
Sex			
Female	79	31 (39.3)	48 (60.8)
Male	187	52 (27.8)	135 (72.2)
Smoking status			
Nonsmoking	139	46 (33.1)	93 (66.9)
Smoking	127	37 (29.1)	90 (70.9)
Tumor type			
Adenocarcinoma	144	44 (30.6)	100 (69.4)
Squamous cell carcinoma	122	39 (32.0)	83 (68.0)
T			
T1 + T2	206	67 (32.5)	139 (67.5)
T3 + T4	60	16 (26.7)	44 (73.3)
N			
N0	130	46 (35.4)	84 (64.6)
N1–N3	136	37 (27.2)	99 (72.8)
Tumor stage			
Stage I	107	41 (38.3)	66 (61.7)[b]
Stage II + III	159	42 (26.4)	117 (73.6)
p53 mutation status			
Absence	179	63 (35.2)	116 (64.8)[c]
Presence	87	20 (23.0)	67 (77.0)

[a] The frequencies of *p53* codon 72 genotypes were 31.2% for *Arg/Arg*, 47.7% for *Arg/Pro*, 21.1% for *Pro/Pro*

[b] $P = 0.04$; stage I group compared with stage II + III group

[c] $P = 0.04$; absence of *p53* mutation status group compared with presence of *p53* mutation status group

patients with *p53* wild-type and *p53* codon 72 *Arg/Arg* genotype were selected as a reference (HR = 1.00), stage I patients with *p53* wild-type and *p53 Pro*72 allele did not show a higher HR for OS (HR = 0.97; 95% CI 0.50–1.87). Stage I lung cancer patients with *p53* mutation and *p53* codon 72 *Arg/Arg* genotype also did not show an increase in HR for OS (HR = 0.97; 95% CI 0.30–3.09). Notably, stage I patients with both *p53* mutation and *p53* codon 72 *Pro/Pro* genotype experienced a 2.66-fold HR for OS (95% CI 1.21–5.85). However, stage II/III lung cancer patients with *p53* mutation and/or *p53* codon 72 *Pro* allele did not show increased HR for OS when compared with those without either *p53* mutation or *p53* codon 72 *Pro* allele.

DISCUSSION

Several studies have been conducted on the association between *p53* mutation and prognosis in NSCLC, but the results have been controversial.[4–6] In addition, the association of *p53* Arg72Pro polymorphism with lung cancer risk has been studied.[9–12] Some previous reports have indicated that the Arg72Pro polymorphism is not associated with lung cancer prognosis.[13–15] Interestingly, our present study showed that the prognostic value of *p53* mutation in stage I lung cancer can be potentially increased by *p53 Pro*72 allele.

TABLE 2 p53 mutation patterns in non-small-cell lung cancer tumors stratified by p53 codon 72 genotypes

p53 mutation type	*Arg/Arg* ($n = 20$; %)	*Arg/Pro* + *Pro/Pro* ($n = 67$; %)
Transition	7 (35.0)	22 (32.8)
A:T → G:C	2 (28.6)	13 (59.1)
G:C → A:T	5 (71.4)	9 (40.9)
Transversion	9 (45.0)	32 (47.8)
A:T → C:G	1 (11.1)	1 (3.1)
G:C → T:A	4 (44.5)	13 (40.6)
G:C → C:G	1 (11.1)	3 (9.4)
C:G → T:A	1 (11.1)	4 (12.5)
C:G → G:C	0 (0.0)	1 (3.1)
T:A → C:G	1 (11.1)	1 (3.1)
T:A → G:C	0 (0.0)	5 (15.6)
T:A → A:T	1 (11.1)	4 (12.6)
Deletion/insertion	4 (20.0)	13 (19.4)

Fischer's exact test was used for the statistical analysis

The prognostic value of *p53* alterations in patients with NSCLC remains controversial.[5,6,21] This controversy may be due to the following reasons: First, the identification of *p53* mutations in clinical samples is generally limited by the assay sensitivity. The most commonly used approach for mutation detection has been the single-strand conformation polymorphism (SSCP) for screening. However, SSCP failed to identify *p53* mutations in 14–38% of tumors in which such mutations were detected by direct dideoxynucleotide sequencing.[22,23] Second, there are technical difficulties associated with microdissection and the possibility of contamination by normal cells during tumor extraction. In our present study, the DNA of tumor cells was isolated by microdissection of the lung tumor tissues, and detection of the *p53* gene mutation was conducted by direct dideoxynucleotide sequencing to validate the data.

p53 mutation that denatures or strongly destabilizes the p53 protein presumably has a greater effect on critical p53 tumor suppressor functions, such as apoptosis and growth suppression.[24] Tomizawa et al.[5] reported that stage I lung cancer patients with *p53* missense mutation at exons 5–8, which is the functional domain of p53 protein, have poorer prognosis than those with *p53* wild type. It has also been demonstrated that *p53* mutation in the L2 + L3 loop, zinc-binding residues, and severe flexible and contact mutations

TABLE 3 Univariate analysis of hazard ratios for overall survival for lung cancer patients of all stages with different clinical parameters, p53 codon 72 genotypes, and mutation status

Variables (*n*)	Median survival (days)	HR (95% CI)
Age, years		1.01 (0.99–1.03)
Sex		
Female (79)	1091	1.00
Male (187)	731	1.87 (1.32–2.65)[a]
Smoking status		
Nonsmoking (139)	813	1.00
Smoking (127)	775	1.23 (0.92–1.64)
Tumor type		
Adenocarcinoma (144)	772	1.00
Squamous cell carcinoma (122)	854	0.92 (0.69–1.23)
T		
T1 + T2 (206)	876	1.00
T3 + T4 (60)	629	1.61 (1.16–2.25)[a]
Nodal metastasis		
N0 (130)	993	1.00
N1–N3 (136)	610	1.94 (1.44–2.61)[a]
Tumor stage		
Stage I (107)	1064	1.00
Stage II + III (159)	688	1.83 (1.34–2.50)[a]
p53 codon 72 genotype		
Arg/Arg (83)	795	1.00
Arg/Pro (127)	924	1.05 (0.74–1.48)
Pro/Pro (56)	738	1.27 (0.85–1.91)
Arg/Pro + *Pro/Pro* (183)	794	1.11 (0.81–1.53)
p53 mutation status		
Wild type (179)	780	1.00
Mutation (87)	821	1.18 (0.87–1.60)

[a] $P < 0.01$

are associated with diminished lung-cancer-related survival.[25] p53 protein or abolishment of p53 protein DNA binding capability by *p53* mutation has been shown to be associated with a greater negative effect on patient survival, whereas missense mutations in regions other than the functional domain have little effect on survival.[6] Therefore, both loss of normal function of wild-type p53 and gain of oncogenic properties of p53 may be possible reasons for decreased survival of early-stage lung cancer patients. Similar to our present study, a prospective study suggested that *p53* mutation predicts poor survival in patients with stage I NSCLC but not in patients with advanced NSCLC.[6] The majority of our stage I patients (23 of 27) with *p53* mutation exhibited missense mutation at exons 5–8, and the significant prognostic value of *p53* mutation was limited to patients with stage I NSCLC. This is probably when tumors progress and become increasingly complex, meaning that it is difficult for a single genetic abnormality to define tumor behavior.

Similar to previous reports, our present study indicated that *p53 Pro*72 allele is associated with an increased frequency of *p53* mutation in lung cancer.[14,26] However, Arg72Pro polymorphism was not associated with specific *p53* mutation pattern, consistent with a previous study.[14] The significance of *p53*-dependent DNA repair may be that suppressed expression leads to decrease in global genomic repair and increase in *p53* mutation observed in association with *Pro*72 allele.[14] In the present study, *p53 Pro*72 allele is associated with advanced tumor stage, but there is no association between *p53* codon 72 genotype and lung cancer prognosis. This may be explained by the *Pro*72 allele being less effective at inducing apoptosis than the *Arg*72 allele, leading to lung cancer patients with *p53 Pro*72 allele at an advanced tumor stage.[7] Patients with breast, lung or head and neck cancer with the *p53* codon

TABLE 4 Hazard ratios for overall survival for different stage lung cancer patients with different p53 mutation status and genotypes

Variables	Stage I			Stage II + III		
	n	Median survival (days)	HR (95% CI)	*n*	Median survival (days)	HR (95% CI)
p53 mutation status						
Wild type	80	1,174	1.00	99	569	1.00
Mutation	27	924	1.79 (1.04–3.10)[a]	60	764	0.85 (0.59–1.23)
p53 codon 72 genotype						
Arg/Arg	41	1,174	1.00	42	548	1.00
Arg/Pro	43	1,093	1.21 (0.67–2.19)	84	667	0.78 (0.51–1.20)
Pro/Pro	23	658	1.62 (0.83–3.17)	33	755	0.94 (0.56–1.55)
Arg/Pro + *Pro/Pro*	66	981	1.34 (0.78–2.29)	117	688	0.83 (0.55–1.24)

[a] $P = 0.04$

TABLE 5 Combined effects of p53 mutation and p53 codon 72 genotype on overall survival among lung cancer patients stratified by tumor stage

p53 codon 72 genotype	Stage I tumor						Stage II/III tumor					
	Wild-type *p53*			*p53* mutation			Wild-type *p53*			*p53* mutation		
	n	Median survival (days)	HR (95% CI)[a]	*n*	Median survival (days)	HR (95% CI)[a]	*n*	Median survival (days)	HR (95% CI)[a]	*n*	Median survival (days)	HR (95% CI)[a]
Arg/Arg	33	1,313	1.00 (Ref.)	9	1,035	0.97 (0.30–3.09)	31	382	1.00 (Ref.)	11	610	0.89 (0.40–1.96)
Arg/Pro + *Pro/Pro*	47	1,093	0.97 (0.50–1.87)	18	760	2.66 (1.21–5.85)[b]	68	667	0.76 (0.46–1.26)	49	794	0.65 (0.38–1.10)

[a] Adjustments for age, gender, smoking status, tumor type and T value

[b] $P = 0.02$

72 *Arg/Arg* genotype were reported to have higher response rates and survival after receiving chemotherapy and radiotherapy.[27–30] Recently, Han et al. found that *p53* codon 72 *Pro/Pro* genotype was more likely to be resistant to cisplatin-based chemotherapy and to predict a worse progression-free survival in advanced NSCLC.[31] This finding suggests that a loss of p53 function may be increased by the *p53 Pro72* allele. Patients with *Pro*72 allele more commonly possessed *p53* mutation, which can partially contribute to lung cancer prognosis. It thus is conceivable that *Pro*72 allele increases the prognostic value of *p53* mutation in stage I lung cancer. In the present study, 55 of 107 stage I patients had information on disease recurrence or tumor metastasis. Among these, patients with *Pro*72 allele (76.2%) showed higher frequency of disease recurrence or metastasis when compared with those with *Arg*/*Arg* genotype (23.8%). Such a result also supports a potential role for *Pro*72 allele in increasing the prognostic value of *p53* mutation in stage I lung cancer.

Our present results reveal that *Pro*72 allele potentially increases the prognostic value of *p53* mutation in stage I lung cancer. However, we could not rule out the limitation of a selection bias and smaller number of study patients by stage-stratified analysis. Further analysis should be done with therapeutic data to distinguish the subgroup of stage I NSCLC patients at increased risk of treatment failure and/or death. Finally, our results should be verified by future studies that include a larger number of stage I patients.

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