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Association of *ANRIL* Polymorphism With Overall Survival in Adult Patients With Hematologic Malignancies After Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract. *Background/Aim: Genetic variations of the non-coding RNA gene, ANRIL, have been associated with human diseases including cancer, type-2 diabetes, and atherosclerosis. In the present study, we investigated the potential associations of select ANRIL single nucleotide polymorphisms (SNPs) with overall survival and other clinical outcomes in adult patients with hematologic malignancies after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Patients and Methods: Genomic DNA was extracted from whole blood samples from 103 adult patients with hematologic malignancies who had received allo-HSCT followed by oral tacrolimus therapy. The genotypes of four select ANRIL SNPs, rs564398, rs1063192, rs2151280, and rs2157719 were determined using qRT-PCR-based genotyping assays. Results: rs2151280 (C->T) in ANRIL was associated with worse overall survival in these patients (CT/CC vs. TT). Contrarily, rs2151280 and the other select ANRIL SNPs were not associated with death at Day-100 after transplantation, the incidence of graft-versus-host disease (GVHD), acute kidney injury (AKI), and neurotoxicity in the study cohort. Conclusion: rs2151280 represents a potential prognostic biomarker for overall survival in adult patients with hematologic malignancies after allo-HSCT.*

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Chromosome 9p21 harbors the *ARF-INK4* locus, which encodes three tumor suppressive proteins, $p15^{INK4B}$ (P15), $p16^{INK4A}$ (P16) and p14ARF (ARF) (1-5). P15 and P16 specifically inhibit cyclin D-dependent kinase 4 (CDK4)-mediated phosphorylation of retinoblastoma protein (pRB), thus regulating cell cycle progression; ARF inhibits the ability of mouse double minute 2 homolog (MDM2) to suppress p53, and consequently promotes apoptosis or cell cycle arrest (4, 5). Recent studies have demonstrated that the non-coding RNA *ANRIL* (also designated as *CDKN2BAS*) is located adjacent to the *INK4-ARF* locus but is transcribed in the antisense direction with respect to *p14ARF* (6-9). *ANRIL* interacts with polycomb repressing complexes 1 and 2 (PRC1 and 2), and negatively regulates the transcription of the entire *INK4-ARF* locus (10-12). Emerging evidence has revealed that some single nucleotide polymorphisms (SNPs) in *ANRIL* are associated with susceptibility to the development of human diseases, including different types of cancer, type-2 diabetes and atherosclerosis (9, 13-26). Specifically, rs564398 has frequently been found in patients with leukemia and esophageal squamous cell carcinoma (ESCC). *ANRIL* rs2151280 has been associated with increased risk of developing lung cancer and basal cell carcinoma (BCC) while rs1063192 and rs2157719 have been correlated with glioma, brain cancer, and ESCC. Additionally, our previous study has demonstrated that

rs2151280 was associated with mRNA expression of *p15*, *p16*, and *ARF* in peripheral blood mononuclear cells (PBMCs) from multiple myeloma patients, and with progression-free survival (PFS) following autologous stem cell transplantation (27). Taken together, these findings imply that *ANRIL* SNPs may impact the development and treatment of human hematologic malignancies.

In this study, we determined the geneotypes of four previously reported cancer-related *ANRIL* SNPs, rs564398, rs1063192, rs2151280, and rs2157719 using whole blood DNA samples from 103 adult patients with hematologic malignancies and evaluated their associations with patient overall survival (OS) following allogeneic hematopoietic stem cell transplantation (allo-HSCT). Our results showed that *ANRIL* rs2151280 alone was associated with OS in the study cohort.

Patients and Methods

Study population. The study population was from a single-institution research protocol to study the pharmacokinetics and pharmacogenomics of tacrolimus and their association with clinical outcomes in allo-HSCT patients. One hundred and three adult patients receiving oral tacrolimus for the prevention of GVHD following allo-HSCT at The Ohio State University Wexner Medical Center were prospectively enrolled following approval from the Institutional Review Board (IRB #2012C0021). Inclusion criteria included age greater than 18 years, patients receiving their first allo-HSCT during the study period, and absence of renal or hepatic dysfunction at baseline. Exclusion criteria included HIV positivity, presence of any medical conditions that would interfere with the patient's ability to provide informed consent, hypersensitivity to any components of tacrolimus, clinical history of any solid organ transplantation, previous chronic use of tacrolimus, and pregnancy.

Genomic DNA extraction and genotyping assays. Genomic DNA was extracted from patient's whole blood sample using the DNAeasy Blood mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNase A was included in the purification procedure to remove potential RNA contamination as recommended by the manufacturer. After quantitation using UV spectrometry, DNA samples were stored at -80°C for future use.

Genotypes of four select *ANRIL* SNPs, rs564398, rs2151280, rs1063192, and rs2157719 were determined as previously described (27).

Statistical analyses. Demographic and clinical characteristics of patients were analyzed using descriptive statistics. Characteristics were compared using Student's *t*-tests, χ^2 tests or Fisher's exact tests where appropriate.

The consistency between the distribution of each *ANRIL* SNP in the study cohort and the Hardy-Weinberg principle and the potential nonrandom allele associations between selected SNPs were analyzed using χ^2 tests.

Patients without known death/alive information were censored at the date of last follow up. Time to death, the time from transplant (Time 0) until death, was analyzed using Kaplan-Meier curves followed by the log-rank test. Multivariate analysis was conducted using the proportional hazard regression model of Cox (Cox PH regression) and a stepwise backward approach was applied for model reduction. The final proportional hazard regression model included factors with *p*-values less than 0.05 and was justified as previously described (28).

Potential associations between *ANRIL* SNPs and the incidence of death at Day-100 post transplant, GVHD, AKI, and neurotoxicity were evaluated using competing risks regression in which death was a competitive risk factor for GVHD, AKI, and neurotoxicity (29). All statistical analyses were conducted using R3.5.0 (R Foundation for Statistical Computing). *p*-Values were two-sided, and unless specified, *p*-values values of less than 0.05 were regarded as statistically significant.

Results

Patients. Table I summarizes the demographic characteristics of 103 patients included in this study. The mean age at transplant of this patient cohort was 55.6 years (±11.8 years), and 46 patients (44.7%) were older than 65 years of age. Additionally, 64 patients (62.1%) were male while 88 patients were Caucasian (85.4%). Sixty-seven patients (65%) were newly diagnosed at time of study entry. Disease diagnosis varied and 41 (49.8%), 8 (7.7%), 8 (7.7%), and 7 (6.8%) patients diagnosed with AML, ALL, DLBCL, and myelofibrosis, respectively. Eighty-nine patients (86.3%) received matched transplants, including matched sibling and matched unrelated donors, while 1 patient received a mismatched unrelated transplant. The remaining patients received cells from umbilical cord donors (13 of 103, 12.8%).

No significant associations were found between age, gender, race, risk score, the ECOG performance score, comorbidity score, baseline SCr, baseline CrCL, and weight-adjusted starting tacrolimus dose. The one exception was a correlation between ECOG performance score and comorbidity score. Patients with higher comorbidity scores (≥3) were more likely to have higher ECOG scores (1 and 2) than patients with low comorbidity scores (0-2) (p=0.007). This is consistent with previous studies showing that the ECOG score significantly increased in cancer patients with comorbidities in comparison with patients without comorbidities (30).

ANRIL SNPs in patients. A number of *ANRIL* SNPs have recently been associated with the development and treatment of human cancers. Four of these SNPs, ss564398, rs2151280, rs1063192, and rs2157719, were investigated in this study due to their reported correlations with human cancers (13-16, 23-27).

Table II summarizes the genotypes of the 4 *ANRIL* SNPs in this study cohort. The minor allele frequencies (MAFs) for these four SNPs were approximately 40%, and the genotype distributions of these SNPs followed the Hardy-Weinberg principle (all *p*-values >0.9) (27). For rs2151280, 31.1% (n=32), 49.5% (n=51), and 19.4% (n=20) of patients were homozygous for the major allele (genotype: CC; ancestral allele), heterozygous (genotype: TC), and

Table I. *Demographic and clinical characteristics of patients included in this study (N=103).*

Characteristics	Values
Age (year)	
Mean (SD)	55.6 (11.8)
Median (IQR)	58.9 (13.9)
>65	46 (44.7%)
Gender	
Female	39 (37.9%)
Male	64 (62.1%)
Race	
Caucasian	88 (85.4%)
Non-Caucasian	15 (14.6%)
Disease type	
Acute myeloid leukemia	41 (39.8%)
Acute lymphoblastic leukemia	8 (7.7%)
Diffuse large B-cell lymphoma	8 (7.7%)
Myelofibrosis	7 (6.8%)
Other	39 (38%)
Disease stage	
Newly diagnosed	67 (65.0%)
Relapsed	36 (35.0%)
Transplant type	
Matched sibling	35 (33.9%)
Matched unrelated	54 (52.4%)
Mismatched unrelated	1 (0.9%)
Umbilical cord	13 (12.8%)
Cell source	
Peripheral blood stem cell transplants	88 (85.4%)
Bone marrow	2 (1.9%)
Umbilical cord	13 (12.8%)
Comorbidity index	
0	14 (13.6%)
1	7 (6.8%)
2	19 (18.4%)
3	21 (20.4%)
4	20 (19.4%)
5 and above	22 (21.4%)
ECOG score	
0	46 (44.7%)
1	55 (53.4%)
2	2 (1.9%)
Risk score	
1	3 (2.9%)
2	16 (15.5%)
3	43 (41.7%)
4	9 (8.7%)
5	15 (14.6%)
6	17 (16.5%)
Baseline CrCL (ml/min)	
Mean (SD)	62.7 (21.7)
Median (IQR)	59.8 (24.6)
Baseline SCr (mg/dl)	
Mean (SD)	0.73 (0.21)
Median (IQR)	0.70 (0.23)
Starting tacrolimus dose (mg/kg)	
Mean (SD)	0.047 (0.011)
Median (IQR)	0.046 (0.007)
AKI	
No	40 (38.8%)
Yes	63 (61.2%)
GVHD grade	
0	52 (50.5%)
1	22 (21.4%)
2	23 (22.3%)
3 and above	6 (5.8%)
Neurotoxicity	
No	80 (77.7%)
Yes	23 (22.3%)
Death during 100 days after transplantation	
No	92 (89.3%)
Yes	11 (10.7%)
Death during the study period	
No	53 (51.5%)
Yes	50 (48.5%)

AKI: Acute kidney injury; CrCL: creatinine clearance; ECOG: the Eastern Cooperative Oncology Group performance score; GVHD: graft-*versus*-host disease; IQR: inter-quartile range (from 1st quartile to 3rd quartile); SCr: serum creatinine; SD: standard deviation.

homozygous for the minor allele (genotype: TT; variant), respectively. Further analyses showed that all four *ANRIL* SNPs were in significant LD (linkage disequilibrium) with each other in our study cohort (all p values <0.01), primarily ascribed to their close chromosomal proximity (27).

rs2151280 is associated with overall survival after allo-HSCT. We then evaluated the potential associations between patient characteristics and OS in the study cohort using univariate and multivariate survival analyses (Table III). Of the 103 patients, 50 (48.5%) died during the study period. The median time to follow-up was 1084 days (date of transplant time was defined as Time 0). Survival analyses using univariate models showed statistically significant associations with OS between age (>65 *vs*. ≤65; HR=2.21, 95%CI=1.26-3.88, p=0.006) and comorbidity score (≥3 *vs*. 0-2; HR=2.09, 95%CI=1.13-3.88, p=0.02). Moreover, *ANRIL* rs2151280 (*CT*/*CC vs*. *TT*) and disease type (AML *vs*. Other) were associated with OS with borderline significance (for rs2151280, HR=0.55, 95%CI=0.29-1.04, p=0.07; for disease type, HR=1.66, 95%CI=0.95-2.89, p=0.075), whereas rs564398 (*CC vs*. *CT*/*TT*) and risk score (1-3 *vs*. ≥ 4) tended to be associated with OS (for rs564398, HR=0.42, 95%CI=0.13-1.35, p=0.15; for risk score, HR=0.63, 95%CI=0.35-1.14, p=0.14). The median time to death for rs2151280 *TT* patients was 379 days. In contrast, the median time to death for rs2151280 *TC*/*CC* patients was >1300 days; thus, median survival was not achieved during the study period (Figure 1). Furthermore, no association was observed between

Table II. *Genotypes of the 4 selected ANRIL SNPs in patients included in this study (N=103).*

SNP	Genotype						Allele			*p*-Value***
	M/M	n_1*	M/m	n_2*	m/m	n_3*	*M***	*m***	MAF	
rs564398	T/T	42	T/C	49	C/C	12	133	73	0.354	0.96
rs1063192	A/A	39	A/G	47	G/G	17	125	81	0.393	0.95
rs2151280	C/C	32	C/T	51	T/T	20	115	91	0.442	0.99
rs2157719	T/T	39	T/C	49	C/C	15	127	79	0.383	0.99

*The number of patients (n) with specific genotypes. M: Major allele; m: minor allele. **The number of patients with specific alleles. MAF: Minor allele frequency. ***χ^2 tests were used to analyze the consistency between the actual genotype distribution and the Hardy-Weinberg principle. *p*-Values of less than 0.05 indicate that the actual genotype distribution significantly deviates from the Hardy-Weinberg principle.

Table III. *Univariate and multivariate analyses of overall survival (OS) of patients included in this study (n=103).*

Characteristics	HR*	95%CI*	*p*-Value**
Univariate			
rs564398 (*CT/TT vs. CC*)	0.42	[0.74, 7.69]	0.15
rs1063192 (*AG/GG vs. AA*)	0.86	[0.65, 2.08]	0.62
rs2151280 (*CT/CC vs. TT*)	0.55	[0.29, 1.04]	0.07
rs2157719 (*CT/TT vs. CC*)	0.54	[0.73, 4.55]	0.20
age (>65 *vs.* ≤65)	2.21	[1.26, 3.88]	0.006
gender (Male *vs.* Female)	0.98	[0.56, 1.74]	0.96
Race (Caucasian *vs.* non-Caucasian)	0.90	[0.40, 2.00]	0.79
ECOG (1/2 *vs.* 0)	1.52	[0.86, 2.69]	0.15
Comorbidity (3 and above *vs.* 0-2)	2.09	[1.13, 3.88]	0.02
Risk score (1-3 *vs.* 4 and above)	0.63	[0.35, 1.14]	0.13
Disease stage (Relapsed *vs.* Newly diagnosed)	0.66	[0.35, 1.22]	0.18
Disease type (AML *vs.* other)	1.66	[0.95, 2.89]	0.075
Starting dose of Tacrolimus per kg weight Increasing by 1 mg/kg)	0.0023	[0.0001, >10,000]	0.67
Baseline creatinine clearance Increasing by 1 ml/min	0.99	[0.98, 1.01]	0.26
Baseline serum creatinine Increasing by 1 mg/dl	0.74	[0.20, 2.69]	0.64
Multivariate***			
rs2151280 (*CT/CC vs. TT*)	0.44	[0.22, 0.85]	0.015
age (>65 *vs.* ≤65)	2.24	[1.27, 3.94]	0.005
Comorbidity (3 and above *vs.* 0-2)	2.57	[1.35, 4.89]	0.004

*HR: Hazard ratio; CI: confidence interval. **p-values from proportional hazard Cox regression analyses. ***The overall *p*-value for this multivariate model was <0.0001.

OS and race, disease stage (relapsed *vs* newly diagnosed), baseline renal function (baseline CrCL), and starting tacrolimus dose (all *p*-values >0.20). In addition, there are no statistically significant differences in the characteristics of the patients harboring rs2151280 *TC/CC* and *TT* genotypes (data not shown).

Further multivariate analyses revealed that rs2151280, age, and comorbidity remained significantly associated with OS in this patient cohort (with *p*-values <0.05). After adjustment for age and comorbidity, the HR between the rs2151280 CT/CC group and the rs2151280 TT group was 0.44 (95%CI=0.22-0.85, *p*=0.015), suggesting that patients with rs2151280 TT genotype have higher risk for death after allo-HSCT. The adjusted HRs for age (Age >65 *vs.* Age ≤65) and comorbidity (≥3 *vs.* 0-2) were 2.24 (95%CI=1.27-3.94, *p*=0.005) and 2.57 (95%CI=1.35-4.89, *p*=0.004), respectively. These findings are consistent with previous studies associating advanced age and presence of comorbidities with poor prognosis (30). Taken together, rs2151280 TT appears to be a potential prognostic factor (in addition to age and comorbidity score) for OS of leukemia patients after allo-HSCT.

Impact of ANRIL SNPs on other clinical outcomes in patients with allo-HSCT. Lastly, we explored the potential associations between the aforementioned *ANRIL* SNPs and the incidence of

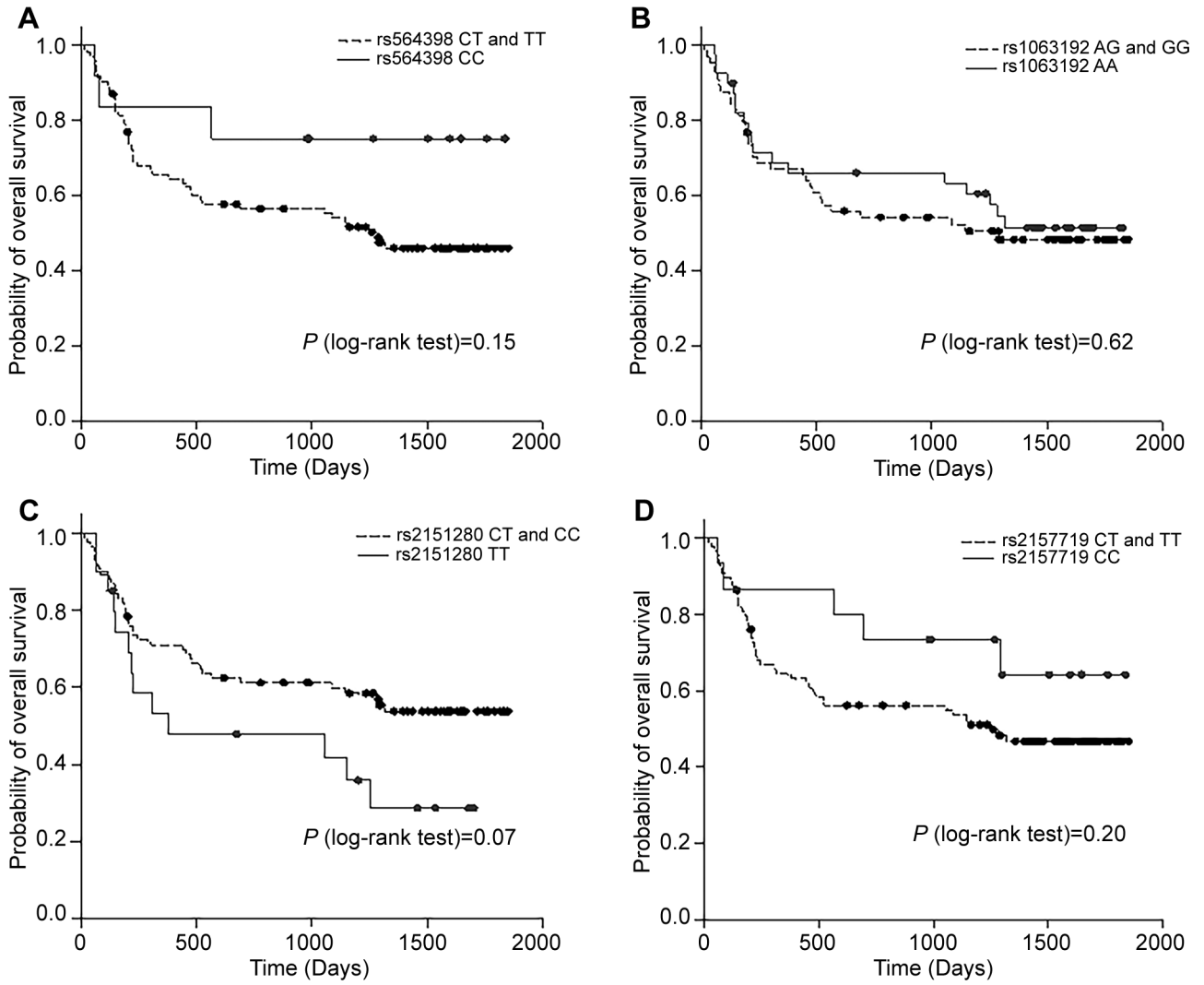

Figure 1. *Kaplan-Meier curves showing the overall survival of 103 patients with different ANRIL genotypes in the four select SNPs following allogeneic hematopoietic stem cell transplantation. Patients with different genotypes were compared using the log-rank tests. A, rs564398 (CT+TT vs. CC); B, rs1063192 (AG+GG vs. AA); C, rs2151280 (CT+CC vs. TT); D, rs2157719 (CT+TT vs. CC).*

death at Day-100, GVHD, AKI, and neurotoxicity in the study cohort. Our results showed that none of the select *ANRIL* SNPs was associated with death at Day-100. Similarly, no statistically significant correlation was observed between these genetic alterations and GVHD or AKI (all *p*-values >0.20). Instead, gender, comorbidity and baseline CrCL were statistically associated with AKI (p=0.015, 0.012, and 0.0006, respectively).

Discussion

In the current study, we revealed that *ANRIL* rs2151280 was significantly associated with OS in adult patients with hematologic malignancies after allo-HSCT. Patients harboring the rs2151280 TT genotype tend to have poorer OS. The underlying mechanism could be ascribed to *ANRIL*-mediated transcription suppression on the entire *ARF-INK4* gene (7, 11, 12). Our previous study had shown that rs2151280 SNP leads to elevated expression of *ANRIL* in PBMCs from multiple myeloma patients, and such increase in *ANRIL* expression simultaneously downregulated the mRNA expression of *p15*, *p16*, *ARF* in PBMCs (27). Presumably, patients homozygous for rs2151280 *TT* would have decreased expression of ARF, which further negatively impacts p53-mediated apoptosis in patients with hematologic malignancies. Similarly, reduced expression of *p16* and *p15* in rs2151280 *TT* patients could down-regulate pRB-mediated cell-cycle control, promoting tumor progression or relapse. Hence, both p53 and pRB pathways in patients harboring

rs2151280 *TT* could be impaired (27, 31), thus influencing the clinical outcomes in adult patients with hematologic malignancies.

There are several limitations in this study. First, the patient cohort was relatively small, and statistical comparisons were performed between two "unbalanced" groups (20 rs2161280 *TT* patients *vs*. 83 rs2151280 *TC+CC* patients). Moreover, the study cohort included diverse hematologic malignancies, such as AML, ALL, DLBCL, and myelofibrosis. The molecular mechanisms underlying these hematologic malignancies as well as their clinical outcomes vary considerably (32, 33). Accordingly, a large cohort of patients would be required to stratify patients based on specific hematologic malignancy. Second, this was a retrospective study and only whole blood DNA specimens were available for genotyping and genetic alteration analyses, precluding analysis of rs2151280 *TT* association with mRNA and protein expression of *p15*, *p16*, and *ARF* in blood. Lastly, the impact of *ANRIL* SNPs other than the select four were not determined. Regardless of these limitations, our results provide a foundation for better understanding the role of genomic variants in *ANRIL* in patients with hematologic malignancies after allogeneic hematopoietic stem cell transplantation.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

MJP and WS contributed to the study design; JL, NS, XZ, JJ, ZV performed experiments and collected data; JL and MP analyzed the data and prepared the manuscript; MJP, WS, MP, and CH contributed to the clinical trial; all co-authors reviewed and approved the final version of the manuscript for submission.

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