

Evaluation of the CD123 Expression and FLT3 Gene Mutations in Patients with Acute Myeloid Leukemia

Akbar Safaei, Ahmad Monabati, Maral Mokhtari, Moeinadin Safavi, Freidoon Solhjoo*

Dept. of Pathology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

Background and Objective: Identification of cytogenetic and molecular changes plays an important role in acute myeloid leukemia (AML) patients. Thus, they are used in classification, prognosis and treatment of the disease. The CD123 expression and FLT3 gene mutations are also the variations that may assist in prognosis and treatment of patients with AML.

Methods: This study was performed on 76 patients as new cases of AML. The correlation between CD123 immunohistochemical (IHC) expression and FLT3 gene mutations with each other as well as morphological, immunophenotypical and cytogenetic factors was studied.

Results: The results represented the CD123 IHC expression in 55.3% and FLT3 gene mutations in 28.9% of cases. We found that 81.3% of patients who had FLT3/ITD gene mutations revealed IHC of CD123 expression ($P=0.019$). The CD123 expression against FLT3 was also correlated with monocytic differentiation in bone marrow blasts ($P=0.031$). There were significant correlations between IHC expression of CD123 and FLT3/ITD mutations with a high percentage of aspirated bone marrow blasts ($P=0.01$ and $P=0.006$, respectively) as well as the lack of CD34 expression in bone marrow blasts ($P=0.007$ and $P=0.021$, respectively).

Conclusion: The CD123 IHC positive AMLs were correlated with certain pathologic features, some of which can be similar with correlations of background mutation of FLT3/ITD; According to the negative predictive value (NPV), 88.2% of CD123 IHC showed FLT3 gene mutation. In addition to its use in targeted therapy, it could be a marker to decide what molecular tests to use in the next steps.

Corresponding information: Freidoon Solhjoo, M.D, Fellow of Molecular Pathology and Cytogenetics, Dept. of Pathology, School of Medicine, Shiraz, Iran, E-mail: solhjoofn@yahoo.com

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Introduction

Acute myeloid leukemia (AML) includes a group of diseases whose common manifestation is increased immature myeloid cells (blast or blast equivalents) in the bone marrow or peripheral blood; generally, more than 20% of the cells include these blasts (1). AML is the most common type of leukemia in adults, and its incidence has remained stable in recent years and shows two age peaks; one in early childhood and another after adulthood (2). The median age of AML is about 65 years (3) and despite rarity it is responsible for a large number of deaths related to cancer

(2). Acute myeloid leukemia has the lowest survival rate among all leukemia, although the survival rate has significantly improved in the younger age group; it remains low in older patients (4).

Genetic abnormalities play a basic role in the diagnosis, treatment and prognosis of acute myeloid leukemia (5) to the extent that in recent decades extensive changes have occurred in their classification (1). Given that about 40-50% of AML has normal karyotype (6), identification of these molecular changes can help to further classification and providing useful prognostic information (5).

The World Health Organization (WHO) classification of AML in 2008, defined 5 new subgroups of recurrent genetic abnormalities, including two categories at the molecular level (7).

Identification of specific molecular abnormalities can help to achieve appropriate targeted therapy (5). Accordingly, the evaluation of molecular mutations at the time of initial diagnosis of AML has become a standard assessment of care that can help the management of patients (8).

One of the factors that could help in this regard is identification of mutations in the gene FLT3 (FMS-like tyrosine kinase 3). FLT3 is a member of the family type III receptor tyrosine kinase (RTK) (9). Its mutations are divided into two types: 1) internal tandem duplication (FLT3/ITD) within or near the second juxta membrane receptor and 2) the point mutations leading to amino acid substitutions in the loop of activating tyrosine kinase domain (mutant FLT3/TKD) (10).

Interleukin (IL)-3 receptor systems include IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors. They regulate growth, differentiation and survival of hematopoietic cells, and conduct immune and inflammatory responses while binding to associated ligands (IL-3, IL-5, GM-CSF) (11). IL-3 receptor system includes cytokine α specific and common β C subunits which are common between three receptors (12). On the other hand, it has been shown that receptor α chain of IL-3 (CD123) is expressed on the myeloid cells and AML blasts (13) while on the normal hematopoietic stem cells they are not presented (14). This overexpression can cause growth of the cells in an environment that has IL-3 levels below the concentration of optimal levels. These signs suggest that increased expression of CD123 which varied in different studies from 30% to 80% (15, 16) and even in one study was up to 95% (13), can promote cell proliferation and induction of leukemia (12); all this leads to strategies that target the CD123 by antibodies (17).

One of the problems in evaluating patients with

AML or other disorders involving bone marrow is accessibility to fresh bone marrow in aspiration; this could create an obstacle to perform molecular tests that require fresh tissue. Of course, in these cases bone marrow biopsy is generally used but due to poor quality of DNA, molecular testing on decalcified and formalin fixed tissues is difficult (18). Fortunately, in these cases we can benefit from IHC in the best way and use it as an alternative to flow cytometry in phenotyping of AMLs (19). Even in some cases the results of some markers in IHC correlated with molecular changes in AMLs has been shown (20). In this study, in addition to identifying the correlation between CD123 IHC expression and FLT3 gene mutation and other pathological views of AML, we tried to answer this question whether we can decide based on the outcome of the CD123 IHC expression for other molecular assessments.

Materials and Methods

This study was performed on patients diagnosed with AML for the first time since June 2015 till June 2016 at Hematopathology Lab affiliated to Shiraz University of Medical Sciences. Those with relapsed previous myeloid leukemia were excluded from the study. The patients had to have adequate samples for IHC and appropriate samples for DNA extraction for PCR and also suitable sample for karyotype cultivation. Total numbers of cases were 76 and IHC, PCR and flow cytometry were performed for all of these patients but culture was not successful for 14 patients and the number of metaphases did not fit enough for complete analysis and reaching the final result.

Immunohistochemistry (IHC) for detection of CD123 was done on EDTA decalcified formalin-fixed, paraffin-embedded (FFPE) tissues of bone marrow biopsy samples (Master diagnostic CD123 antibody) (7G3). To be considered as positive, >20% of cells had to be reactive as cut off (Figure 1). The IHC results were evaluated by two pathologists separately and in cases with conflict between them, they jointly reviewed to reach the final verdict.

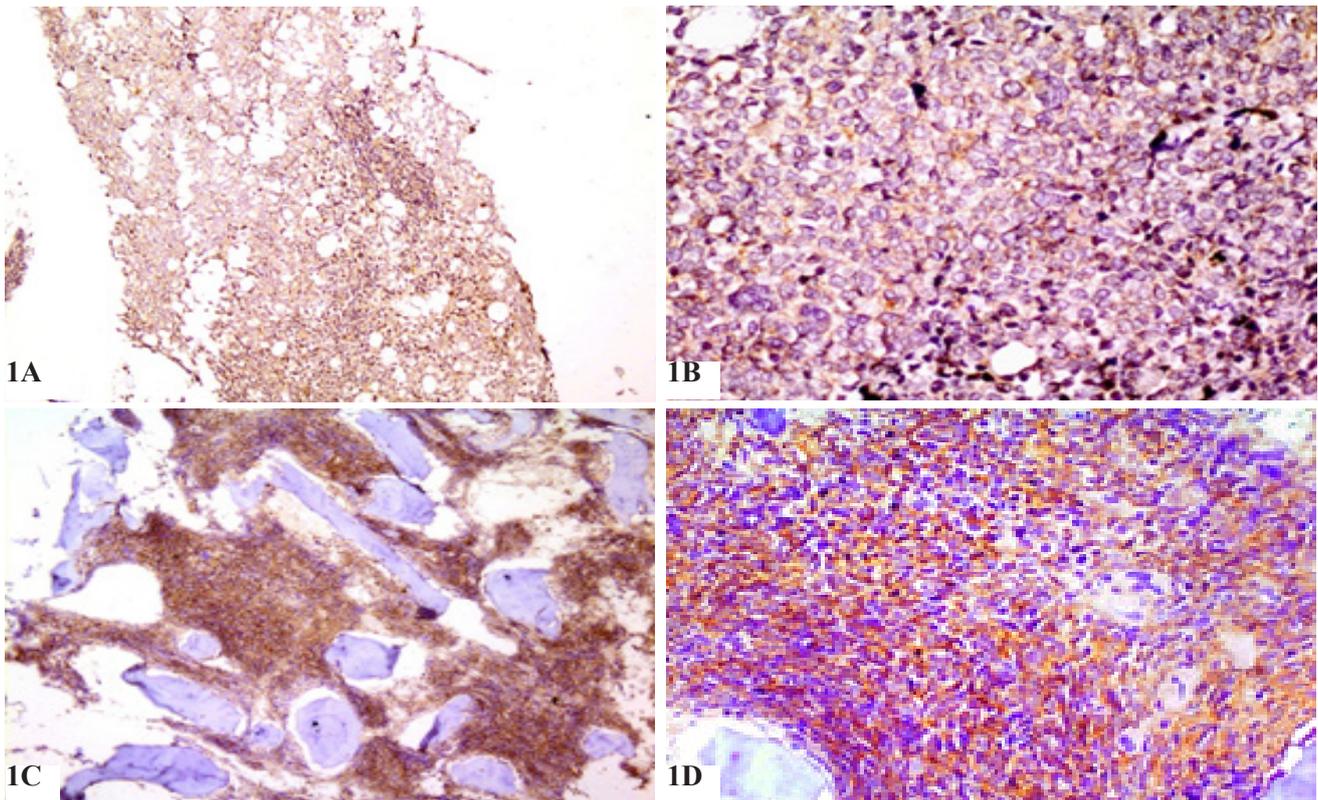


Figure 1. CD123 IHC staining. A and B show low and high power of weak cytoplasmic and/or membranous CD123 IHC staining. C and D show low and high power of strong cytoplasmic and/or membranous CD123 IHC staining. (Counterstain with hematoxylin. A, C \times 100 and B, D \times 400)

FLT3 gene mutations analysis was carried out on genomic DNA extracted from EDTA anticoagulated bone marrow aspiration based on the phenol-chloroform extraction and precipitation with ethanol. Two major types of mutations were checked, ITD (insertion of 3 nucleotide repeats) and D835 (substitution of phenylalanine instead of aspartic acid at position 835aa). The sequence of primers used for FLT3/ITD mutation were as follow: F-5'-GCAATTTAGGTAT-GAAAGCCAGC-3' and R-5'-CTTTCAGCATTTT-GACGGCAACC-3'. PCR was performed on Kyratec PCR Cycler in a total volume of 25 μ l. PCR mixture included 2.5 μ l of 10X buffer, 1 μ l of 50 mM $MgCl_2$, 0.5 μ l of 10 mM deoxynucleoside triphosphates, 0.5 μ l of 3 U/ μ l Taq polymerase, 1 μ l of each primer (10 μ M) and 3 μ l of the extracted DNA template. The reactions were performed using the following program: 5 min denaturation at 95°C, 35 amplification cycles of 95°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec, followed by a final extension of 5 min at 72°C. The

PCR product was run on 3% agarose gel. The wild type FLT3 showed only one single sharp band at 328 bp. The mutant cases had another larger band which was exact multiplication of FLT3 (Figure 2A).

The sequences of primers used for FLT3/TKD D835 were: F5'—CGCCAGGAACGTGCTTGTCA3' and R5'—TCAAAAATGCACCACAGTGAGTGC3'. The PCR mix and program was completely the same as above. Ten μ l of PCR product was treated overnight at 37°C with 1 μ l of Eco RV restriction enzyme and 3 μ l of 10X Tango buffer in a total volume of 30 μ l. The final product was run on 2% agarose gel. The wild type cases revealed a band of 196 bp before digestion and two bands of 129 and 66 bp after digestion. The mutation can change the restriction site and they had an intact 196 original band preserved (Figure 2B).

Flow cytometry analysis of patients' samples using Facscalibur (BD) was done by a comprehensive panel covering all hematopoietic lineages. Antibod-

ies used for monocytic differentiation were CD14, CD64, CD4, and CD33 in combination with CD45 (Dako). Data was analyzed by Cell Quest Pro Version 6 software. Monocytic differentiation was confirmed by bright expression of CD33 on monocyte gate in SSC/CD45 and by expression of CD14 and/or CD64.

For karyotyping of bone marrow aspiration, the samples were cultivated on RPMI 1640 to which FBS (fetal bovine serum), antibiotics (penicillin/streptomycin) and L-glutamine were added. After synchronization with Uridine, Fluorodeoxyuridine and then Thymidine, cultivated cells were harvested by colcemide and hypotonic solution (KCL) and fixative (methanol: acetic acid 3: 1). At the end of the process

which lasted for about 30 hr, the smears were prepared with thermal method and then analyzed using the Cytovision 4.2 software. Sixty two patients whose karyotype were analyzed perfectly, were classified according to WHO 2008 classification and put in different risk group stratifications based on the cytogenetics.

Statistical analysis

Statistical analysis was performed using SPSS software version 18. Chi-square test and Fisher's exact test were used to compare qualitative variables. T-test was used to compare quantitative variables. $P < 0.05$ was considered as statistically significant.

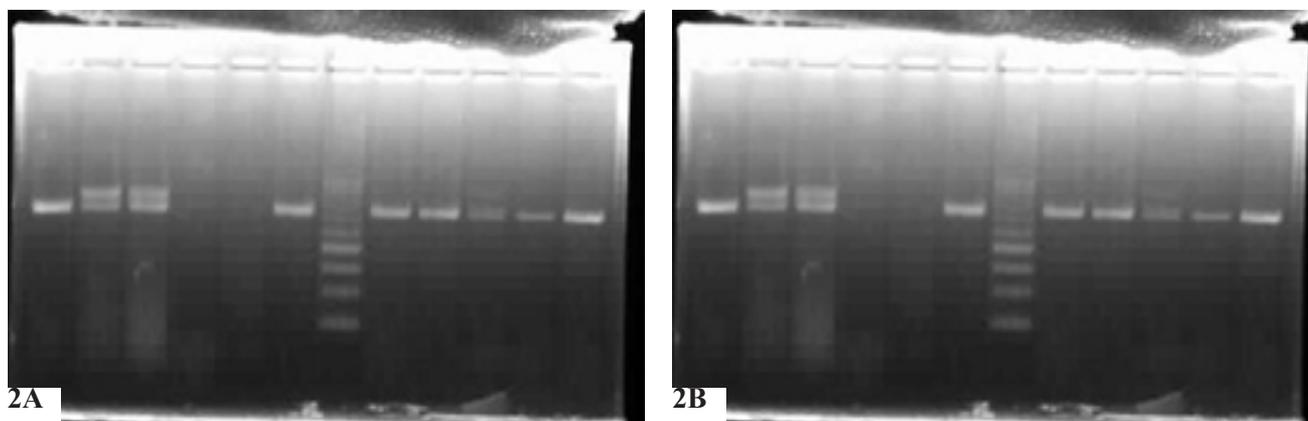


Figure 2. A) FLT3/ITD gel electrophoresis shows from left to right, lanes 1,6,8,9,10,11,12: wild type. Lanes 2,3: mutant. Lanes 4,5: negative controls, lane 7: ladder. B) FLT3/TKD gel electrophoresis shows from left to right, lanes 1,2,3: PCR products before treatment with restriction enzyme. Lanes 4,5,6: PCR products after treatment with restriction enzyme that reveals mutation (heterozygous) in lane 5.

Results

This study was performed on 76 patients (47 male and 29 female). The age range of the patients under the study varied from 3 months to 85 years and their median age was 44.5 years (45 years for men and 43.5 for women). Clinical data of the patients are summarized in Table 1.

Weak to strong expressions as a cytoplasmic and/or membranous CD123 (Figure 1) was seen in 55.3% (42/76) of patients. The FLT3 gene mutations were seen in 28.9% (22/76) of cases, 14 patients had FLT3/ITD, 6 patients with FLT3/TKD and 2 patients had

both mutations. Table 2 summarizes the patients' information and relation of classifications based on the WHO 2008 risk group stratification, immunophenotype with CD123 expression, and FLT3/ITD and FLT3/TKD gene mutations. As can be seen in Table 2, CD123 expression has been observed in a wide range of AML subtypes in the WHO 2008 classification categories but it was not statistically correlated with any of them, so as for none of the cytogenetic risk groups. The average percentage of blasts in the bone marrow aspirates was 71% in cases with expression of CD123 and 58% in cases without expression ($P=0.01$).

Table 1. Clinical data of the patients

Symptom and Sign	Male (N=47)(%)	Female (N=29)(%)	Total (N=76)(%)
Fatigue and lethargy	31 (66)	23 (79)	54 (71)
Fever	22 (47)	18 (62)	40 (53)
Weight loss	23 (49)	16 (55)	39 (51)
Unusual bleeding	17 (36)	21 (67)	38 (50)
Pale skin	12 (26)	15 (52)	27 (36)
Frequent infection	12 (26)	12 (41)	24 (32)
Loss of appetite	10 (21)	14 (48)	24 (32)
Night sweat	11 (23)	12 (41)	23 (30)
Headache	9 (19)	11 (38)	20 (26)
Shortness of breath	8 (17)	11 (38)	19 (25)
Feeling cold	5 (11)	12 (41)	17 (22)
Sleepiness	5 (11)	7 (24)	12 (16)
Confusion	6 (13)	3 (10)	9 (12)
Bone pain	5 (11)	4 (14)	9 (12)
Vomiting	2 (4)	2 (7)	4 (5)
Blurred vision	2 (4)	0 (0)	2 (3)
Skin nodules	1 (2)	0 (0)	1 (1)
Enlarged lymph nodes	1 (2)	0 (0)	1 (1)
Swollen gums	0 (0)	1 (3)	1 (1)

Table 2. CD123 IHC expression, FLT3/ITD and FLT3/TKD mutational status by AML subtype, cytogenetic risk group and pathologic features

AML Subtype*	CD123			FLT3/ITD			FLT3/TKD		
	Positive (n=42)(%)	Negative (n=34)(%)	P	Positive (n=16)(%)	Negative (n=60)(%)	P	Positive (n=8)(%)	Negative (n=68)(%)	P
AML, NOS	20 (59)	9 (32)	0.059	10 (77)	19 (39)	0.014	5 (63)	24 (44)	0.46
AML with recurrent cytogenetic abnormalities	8 (23)	11 (39)	0.18	2 (15)	17 (35)	0.18	2 (25)	17 (31)	0.71
inv (16)	1	1		0	2		0	2	
inv (3) or t (3;3)	0	1		0	1		0	1	
t (15;17)	6	4		2	8		2	8	
t (8;21)	1	2		0	3		0	3	
t (6;9)	0	1		0	1		0	1	
t (9;11)	0	2		0	2		0	2	
AML with myelodysplasia-related changes	4 (12)	7 (25)	0.17	1 (8)	10 (20)	0.29	1 (12)	10 (19)	0.67
Therapy-related AML	2 (6)	1 (4)	1	0 (0)	3 (6)	1	0 (0)	3 (6)	1
Risk Group*									
Favorable	8 (23)	7 (25)	0.89	2 (15)	13 (27)	0.4	2 (25)	13 (24)	0.95
Intermediate	21 (62)	13 (46)	0.22	10 (77)	24 (49)	0.047	5 (62)	29 (54)	0.64

AML Subtype*	CD123			FLT3/ITD			FLT3/TKD		
	Positive (n=42)(%)	Negative (n=34)(%)	<i>P</i>	Positive (n=16)(%)	Negative (n=60)(%)	<i>P</i>	Positive (n=8)(%)	Negative (n=68)(%)	<i>P</i>
Normal karyotype	18 (53)	9 (32)	0.1	9 (69)	18 (37)	0.036	5 (62)	22 (41)	0.25
Abnormal karyotype	3 (9)	4 (14)	0.69	1 (8)	6 (12)	0.64	0 (0)	7 (13)	0.28
Adverse	5 (15)	8 (29)	0.18	1 (8)	12 (24)	0.19	1 (13)	12 (22)	0.52
Pathologic feature									
Median percentages of blast in aspiration	71%	58%	0.010	78%	62%	0.006	71%	65%	0.44
Monocytic differentiation	12(29)	3(9)	0.031	4(25)	11(18)	0.552	3(37)	12(18)	0.18
CD34 negative blasts	24(57)	9(26)	0.007	11(69)	22(37)	0.021	5(62)	28(41)	0.28

AML: acute myeloid leukemia, inv: inversion, NOS: not otherwise specified, t: translocation

*62 patients who had successful culture included in this analysis (missing cultures excluded from this analysis). Bold numbers highlight statistical significance

The rate of 42.9% of CD123 positive patients was also positive for CD34 in flow cytometry while 57.1% were CD34 negative. These percentages were 73.5% and 26.5% for CD123 negative patients, respectively. This means that CD123 expression is reversely associated with CD34 expression ($P=0.007$). Twelve out of 15 patients with AML with monocytic differentiation were CD123 positive. That was not seen for other lineages, this also represents a significant correlation between CD123 positive AML and presence of monocytic differentiation ($P=0.031$).

Patients who had FLT3 gene mutations were CD123 positive in 81.8% of cases while this was only 44.4 for those with wild type FLT3 ($P=0.003$). This was due to FLT3/ITD mutations (0.019). The mutation of FLT3/TKD had no correlation with CD123 expression ($P=0.28$). On the other hand, unlike CD123, FLT3/ITD was associated with AML, NOS ($P=0.02$) and intermediate cytogenetic risk group ($P=0.04$) with normal karyotype ($P=0.03$). Another finding which is worth mentioning is the high negative predictive value of CD123 for FLT3 mutation (NPV=88.2%). In addition, FLT3 gene mutations correlated with some pathological aspects in AML patients and of course some of them held in common with CD123 IHC expression, i.e. high percentage of blasts in bone marrow

aspiration ($P=0.005$) and CD34 negativity ($P=0.005$). This correlation was seen with ITD ($P=0.006$ and $P=0.021$, respectively) but not TKD mutation ($P=0.43$ and $P=0.28$, respectively). Unlike CD123, FLT3 gene mutations did not show correlation with monocytic differentiation ($P=0.091$).

Discussion

At first glance, what attracted the attention was the lower median age of the patients (44.5 years) compared to the literature (65 years) (2). This difference could have several reasons and perhaps might be due to racial and environmental characteristics of the population under the study or even the number of cases in this study. However, in some studies conducted on the population of non-Western societies, the lower median age was found. For example, Bekadja et al. (21) reported a median age of 44.7 years in a study on patients with AML in Algeria. All this indicates the necessity of large-scale epidemiologic studies in Iran, especially in the southern part.

In our study, it was shown that CD123 IHC expression and mutant FLT3 (FLT3/ITD) had significant correlation. In some early studies, pathological features of CD123 positive AML were examined by flow cytometry, but no attention paid to the molecular changes (22). Subsequent works found relationship

between FLT3 gene mutations and IHC expression of CD123 in AML patients (5, 23) but another study found no correlation (24). Since the assessment in that study (24) has been conducted on CD34 positive blasts and given that CD123 expression is higher in CD34 negative blasts, we may justify this discrepancy in the selection of patients for the study.

In our study, besides the correlation of FLT3/ITD with intermediate cytogenetic risk group and normal karyotype, CD123 expression and FLT3 gene mutation correlated with some pathological features in AML patients such as high percentage of blast counted in primary bone marrow aspiration. Such results have also been shown in some previous studies (5). In another study, a high percentage of bone marrow blasts was seen in AML patients with the presence of both NPM and FLT3 gene mutations (25).

The other finding of our study was the correlation between the expression of CD123 and FLT3 gene mutation with CD34 negativity as well as the correlation between the expression of CD123 and monocytic differentiation in bone marrow blasts. Rollins-Raval et al. (5) achieved relatively similar results except that in their study, FLT3 gene mutation did not correlate with CD34 negativity. It should

be noted that in our study, capillary electrophoresis was not used for the evaluation of PCR product to assess FLT3/ITD gene mutation; it is likely that we have lost some mutations with little increase in the number of bps which was not revealed on agarose gel electrophoresis.

Conclusion

The CD123 antibody usage in diagnosis of patients with AML is growing. Due to its association with molecular changes such as FLT3, and perhaps other gene mutations, it can be used as a guide marker for molecular testing. The CD123 expression evaluation may be helpful in predicting gene mutations and phenotype as well as prognosis.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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