Emergence of Concurrent Multiple *EGFR* Mutations and *MET* Amplification in a Patient With *EGFR*-Amplified Advanced Gastric Cancer Treated With Cetuximab

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INTRODUCTION

Chromosomal instability is one of the characteristics of gastric cancer (GC)[1,2] associated with frequent amplifications of receptor tyrosine kinase (RTK)-related genes. Epidermal growth factor receptor (EGFR) is a transmembrane RTK, and its dysregulation is caused by altered *EGFR* gene that drives cancers.[3] Approximately 5%-10% of patients with GC have *EGFR* amplification, which indicates a poor prognosis.[4-6] Several studies suggested the benefit of anti-EGFR therapy for GC with high *EGFR* copy number (CN).[7,8] However, randomized trials failed to demonstrate the survival benefit of anti-EGFR treatments for advanced GC without patient enrichment.[9,10]

Intratumoral heterogeneity and concurrent genomic alterations in downstream molecules or other signaling pathways have been suggested as possible resistance mechanisms to EGFR-targeted therapies for GC.[11] Circulating tumor DNA (ctDNA) analysis is a useful method to detect genomic alterations of tumor cells throughout the body and to identify concurrent heterogeneous resistance mechanisms possibly missed in single-lesion tumor biopsies.[12,13] Using serial ctDNA analysis, Maron et al[14,15] identified acquired genomic alterations in patients with *EGFR*-amplified GC, including emergence of *EGFR*-negative clones; *PTEN* deletion; *KRAS* amplification/mutation; *NRAS*, *MYC*, and *ERBB2* amplification; and *GNAS* mutations.

We present a patient with *EGFR*-amplified GC who acquired substantial numbers of *EGFR* mutations and *MET* amplification during the cetuximab treatment as detected by serial ctDNA sequencing. Furthermore, genomic characteristics of GC with ctDNA *EGFR* amplification are summarized. The patient provided written informed consent for the presentation of anonymized clinical information. Our study and reporting of this patient were performed after approval by the institutional review board at the National Cancer Center Japan, our institution.

CASE REPORT

A 42-year-old man underwent distal gastrectomy with lymph node dissection for localized human epidermal growth factor receptor-2–negative GC. Histopathology showed poorly differentiated adenocarcinoma invading into submucosa or deeper with a minor component of moderately to well-differentiated adenocarcinoma within the lamina propria. He received adjuvant chemotherapy with S-1 plus docetaxel followed by S-1 for 1 year. However, he developed multiple lymph node and bone recurrences after 1 month. Irinotecan plus cisplatin and nab-paclitaxel plus ramucirumab were administered; however, the disease progressed within 1 month on each treatment. Nivolumab was initiated; nevertheless, the patient was admitted because of disseminated intravascular coagulation (DIC), with decreased platelet count and fibrinogen level.

ctDNA sequencing was performed using Guardant360 assay (Guardant Health Redwood City, CA), which detects genomic alterations in 74 genes using ctDNA, before first-line chemotherapy. ctDNA sequencing identified *EGFR* (plasma CN [pCN], 107.9) and *BRAF* (pCN, 2.9) amplification and *RHOA* and *TP53* point mutations (Table 1). Immunohistochemistry (IHC) analysis showed a strong EGFR expression in 70% of tumor cells (Figs 1A and 1B) in archival surgical samples, whereas mismatch repair proteins were proficient, and chromogenic in situ hybridization for EBV-encoded RNA was negative.

Based on ctDNA sequencing, off-label use of cetuximab, a monoclonal antibody for EGFR, was initiated. Eight days after the treatment initiation, DIC rapidly improved. The positron emission tomography–computed tomography (PET-CT) scan on day 21 showed a significant reduction of [^{18}F]fluorodeoxyglucose uptake in multiple bone metastases, and the serum carcinoembryonic antigen and carbohydrate antigen 19-9 (CA 19-9) levels markedly decreased (Figs 1C and 1D). However, 2 months after the

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TABLE 1. Alterations Detected by ctDNA Sequencing at Each Time Point

Alteration	Pre-Cetuximab		Post-Cetuximab		Post-Afatinib/Crizotinib	
Amplification	**Alteration**	**pCN**	**Alteration**	**pCN**	**Alteration**	**pCN**
	EGFR amplification	107.9	*EGFR* amplification	8.9	*EGFR* amplification *MET* amplification	58.9
	BRAF amplification	2.9	*MET* amplification	20.0		41.7
Actionable mutation	**Alteration**	**VAF (%)**	**Alteration**	**VAF (%)**	**Alteration**	**VAF (%)**
	TP53 R342[a]	52.3	*TP53* R342[a]	51.7	*TP53* R342[a]	73.2
	RHOA Y42S	32.3	*RHOA* Y42S	34.5	*RHOA* Y42S	41.4
			EGFR G465V	6.6	*EGFR* G465V	30.8
			EGFR G465E	2.1	*EGFR* S464L	4.1
			EGFR S464L	1.6	*EGFR* G465E	2.9
			EGFR R222C	1.4	*EGFR* G465R	2.0
			EGFR G465R	1.2	*EGFR* N771_H773dup	0.02
			EGFR G719D	0.03	*ATM* Splice Site SNV	0.2
			EGFR N771_H773dup	0.01	*BRAF* V600E	0.03
VUS or synonymous mutation	**Alteration**	**VAF (%)**	**Alteration**	**VAF (%)**	**Alteration**	**VAF (%)**
			EGFR G449R	1.0	*EGFR* V461V	0.7
			EGFR R494K	0.9	*EGFR* D458N	0.5
			EGFR E455E	0.8	*EGFR* E496K	0.5
			EGFR V461V	0.8	*EGFR* S442R	0.5
			EGFR L453L	0.5	*EGFR* S447Y	0.5
			EGFR Q486E	0.5	*EGFR* T474I	0.5
			EGFR E455D	0.3	*EGFR* E602Q	0.4
			EGFR S492_N493del	0.3	*EGFR* I491del	0.4
			EGFR A1013V	0.2	*EGFR* Q435E	0.4
			EGFR A202V	0.2	*EGFR* E455D	0.3
			EGFR V461V[a]	0.2	*EGFR* E455E	0.3
			EGFR V461V[a]	0.2	*EGFR* L438V	0.3
			EGFR E602Q	0.1	*EGFR* L443V	0.3
			EGFR G465G	0.1	*EGFR* R494I	0.3
			EGFR N493_E496delinsK	0.01	*EGFR* R494K	0.3
			ROS1 P1721T	0.1	*EGFR* S437C	0.3
					EGFR V461V	0.3
					EGFR A439E	0.2
					EGFR A439T	0.2
					EGFR D460H	0.2
					EGFR L443L	0.2
					EGFR L453L	0.2
					EGFR S447F	0.2
					EGFR S452F	0.2
					EGFR W477C	0.2
					EGFR A202V	0.1
					EGFR G465A[a]	0.1
					EGFR G465A[a]	0.1
					AR I673fs	0.07

Abbreviations: EGFR, epidermal growth factor receptor; pCN, plasma copy number; VAF, variant allelic frequency; VUS, variant of unknown significance.
[a]Different nucleotide variants but with the same amino acid sequence.

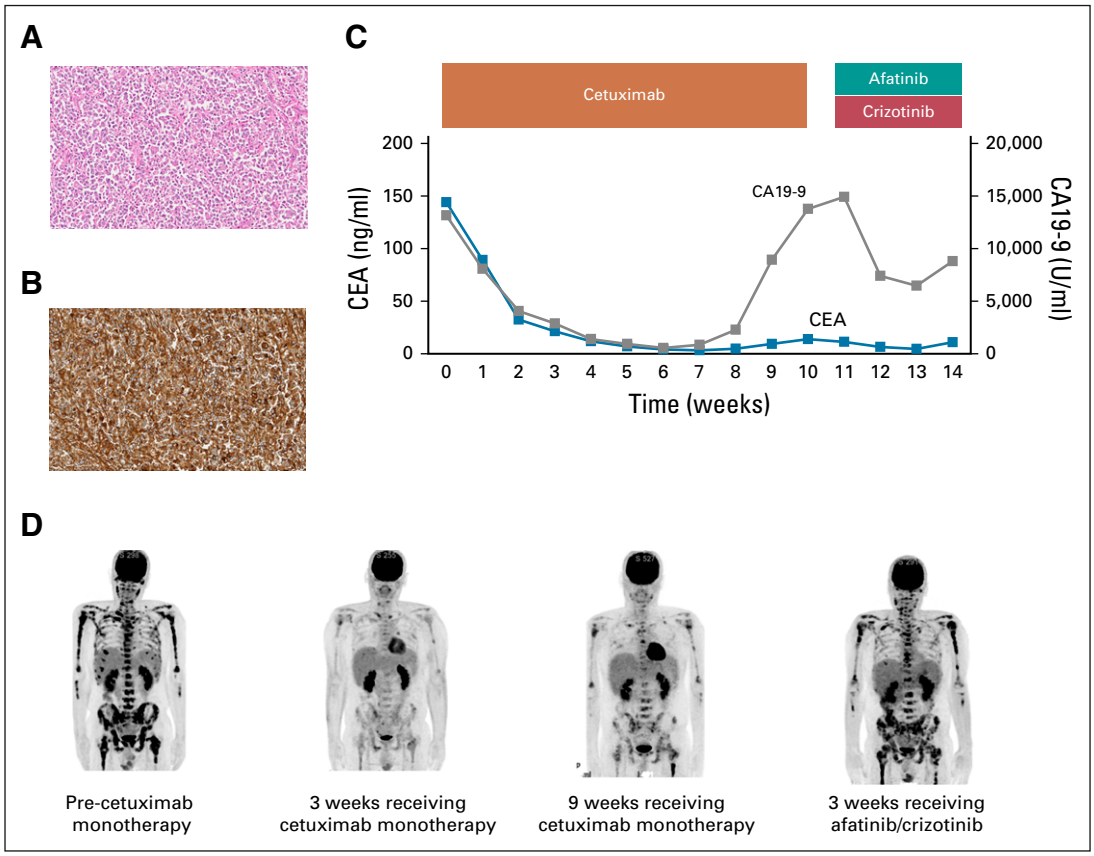

FIG 1. Clinical presentation. (A) Hematoxylin and eosin–stained biopsy specimen of the primary tumor. (B) Immunohistochemistry analysis showing strong epidermal growth factor receptor (EGFR)-positive staining. (C) Course of tumor markers (carcinoembryonic antigen [CEA] and carbohydrate antigen 19-9 [CA 19-9] while receiving treatment with cetuximab and afatinib/crizotinib. (D) Whole-body positron emission tomography–computed tomography scan showing multiple bone metastases pre-cetuximab monotherapy; reduction of [^{18}F]fluorodeoxyglucose (^{18}F-FDG) uptake in bone metastases 3 weeks after the cetuximab monotherapy; reincreased ^{18}F-FDG uptake 9 weeks after the cetuximab monotherapy; and progression 3 weeks after afatinib/crizotinib.

initiation of cetuximab, the patient complained of fatigue, and the serum CA 19-9 increased (Fig 1C). The PET-CT scan on day 63 confirmed bone metastasis progression (Fig 1D).

ctDNA analysis using Guardant360 during disease progression revealed decreased *EGFR* pCN (to 58.9), emergence of 22 new *EGFR* mutations, and *MET* amplification (Figs 2A and 2B; Table 1). *EGFR* mutations extended from the furin-like domain to beyond the tyrosine kinase domain and included four known pathogenic mutations in the extracellular domain (ECD; Fig 2C).

In an attempt to target acquired *EGFR* mutations and *MET* amplification, combination therapy with afatinib and crizotinib was initiated,[16] which led to temporary pain relief and decreased serum CA 19-9 levels but was discontinued on day 35 because of progression of bone metastases (Figs 1B and 1C). ctDNA analysis at that time showed the presence of additional *EGFR* mutations and increased *EGFR* and *MET* pCN (Table 1). The patient died of disease progression 2 months after the discontinuation of afatinib and crizotinib.

CTDNA PROFILE OF *EGFR*-AMPLIFIED GC

To assess the incidence and genomic profiling of GC with *EGFR* amplification in ctDNA, ctDNA results of GC in our institution were reviewed. *EGFR* amplification was identified in 26 (18%) of 148 patients with metastatic GC between September 2018 and December 2019. Among them, *EGFR* pCN was bimodally distributed, with the majority (20; 77%) having low pCN, ranging from 2.2 to 3.4, and the remainder (6; 23%) having pCN of ≥ 3.5, which corresponds to the 90th percentile for *EGFR* pCN across the Guardant360 database for all tumor types (Fig 3A). Bimodal distribution of *EGFR* pCN implies that ctDNA sequencing may identify not only homogeneous focal *EGFR* amplification but also heterogeneity with mixed amplified and nonamplified clones or aneuploidy-associated CN gains, representing low pCN amplifications. Indeed, compared with sample databases tested using tissue-sequencing (GI-SCREEN, our nationwide tissue genotyping study using the Oncomine comprehensive assay [Thermo Fisher Scientific, Waltham, MA], and the Cancer

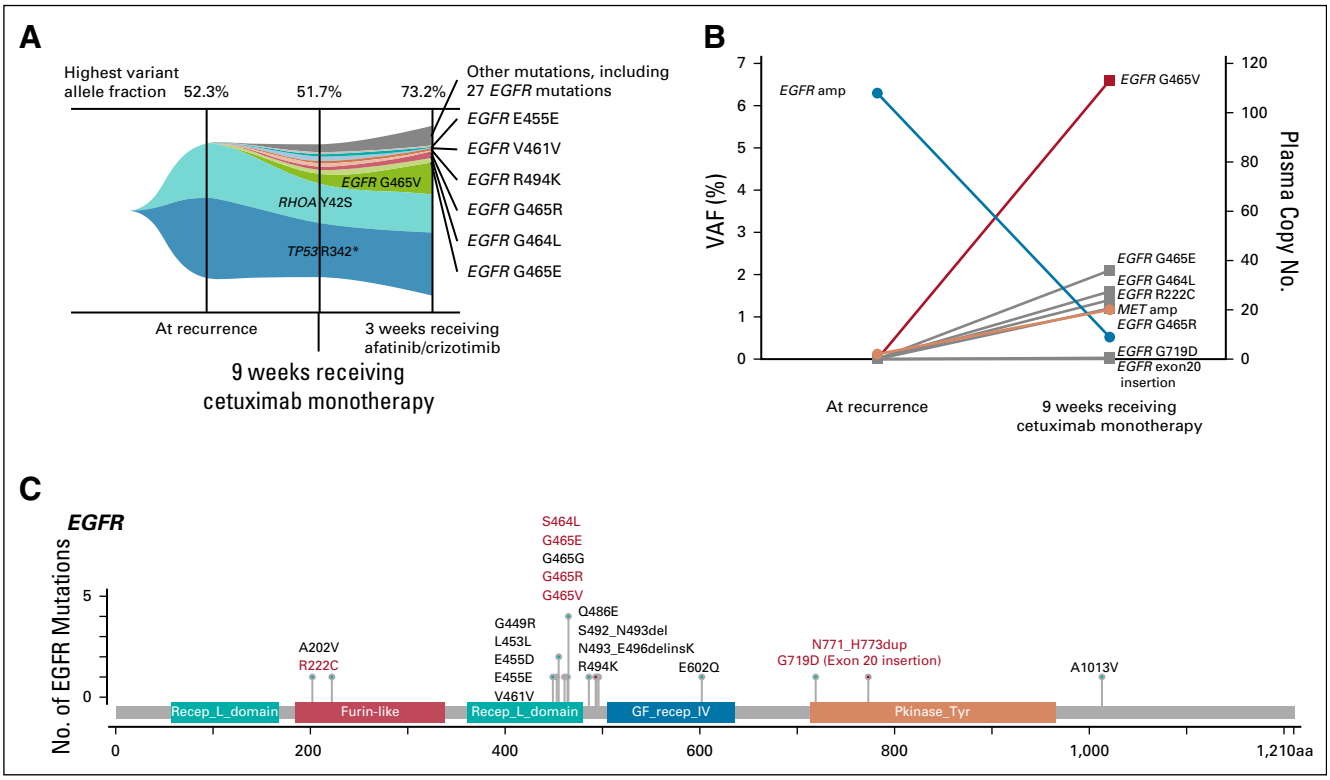

FIG 2. Concurrent emergence of epidermal growth factor receptor (*EGFR*) mutations and *MET* amplification. (A) Tumor-response map showing increased genomic diversity through anti-EGFR therapy. (B) Decreased plasma copy number of *EGFR* and emergence of multiple pathogenic *EGFR* mutations and *MET* amplification with cetuximab treatment. (C) Acquired mutations in *EGFR* domains after cetuximab monotherapy. Actionable variants are highlighted in red. Actionable alterations were annotated using Catalogue of Somatic Mutations in Cancer and genomic visualization tools from cBioPortal were used. VAF, variant allelic frequency.

Genome Atlas [TCGA], a publicly available database[1]), the frequency of all *EGFR* amplifications was significantly greater in ctDNA, whereas the frequency with high pCN (≥ 3.5) *EGFR* amplification more closely matched the findings from tissue databases (all-in ctDNA *EGFR*, 15%; only high pCN *EGFR*, 4%; GI-SCREEN, 4%; and TCGA, 5%; Fig 3B). We also compared the number of acquired *EGFR* mutations between our patient with GC and those with 128 metastatic colorectal cancer (mCRC) patients after disease progression with an anti-EGFR therapy. *EGFR* mutations were detected in 37 patients, including 16 patients with an *EGFR* amplification. The number of *EGFR* mutations (range, 1-7; Fig 3C) or known actionable *EGFR* mutations (range, 1-6; Fig 3D) were lower than those seen in our GC patient with 22 *EGFR* mutations, including seven known actionable mutations.

DISCUSSION

We present a patient with *EGFR* amplification who acquired *EGFR* mutations. This patient had markedly high *EGFR* pCN (107.9) in ctDNA, suggesting *EGFR* focally amplified disease, confirmed by tissue IHC. Baseline ctDNA sequencing also showed not only concurrent *TP53* mutation but also *RHOA* mutation, reflecting mixed histologic findings.[1,17] Our ctDNA genomic profiling study shows that GC with ctDNA *EGFR* amplification can be divided into two clusters according to pCN. The similar frequency of high *EGFR* pCN according to ctDNA and *EGFR* amplification according to tissue analysis suggests that the 90th percentile cutoff for ctDNA most likely enriches for patients with *EGFR* focally amplified disease, although the cutoff for high *EGFR* pCN needs to be confirmed in a larger cohort because the pCN can be affected by several factors, including disease burden.[13] This interpretation is also supported by a previous report on patients with GC treated with an anti-EGFR antibody-containing regimen, in which responders had a median *EGFR* pCN of 33.9 compared with 2.5 in nonresponders.[15]

This patient responded to cetuximab once; however, the disease progressed after only 2 months with numerous acquired mutations throughout *EGFR*, including the ECD and *MET* amplification. These heterogeneous resistance alterations might be suggested to be associated with the histopathologic heterogeneity shown in the primary tumor. *EGFR* ECD mutations are known to indicate anti-EGFR therapy resistance in mCRC due to the interference with binding of anti-EGFR antibodies.[18,19] The failure of

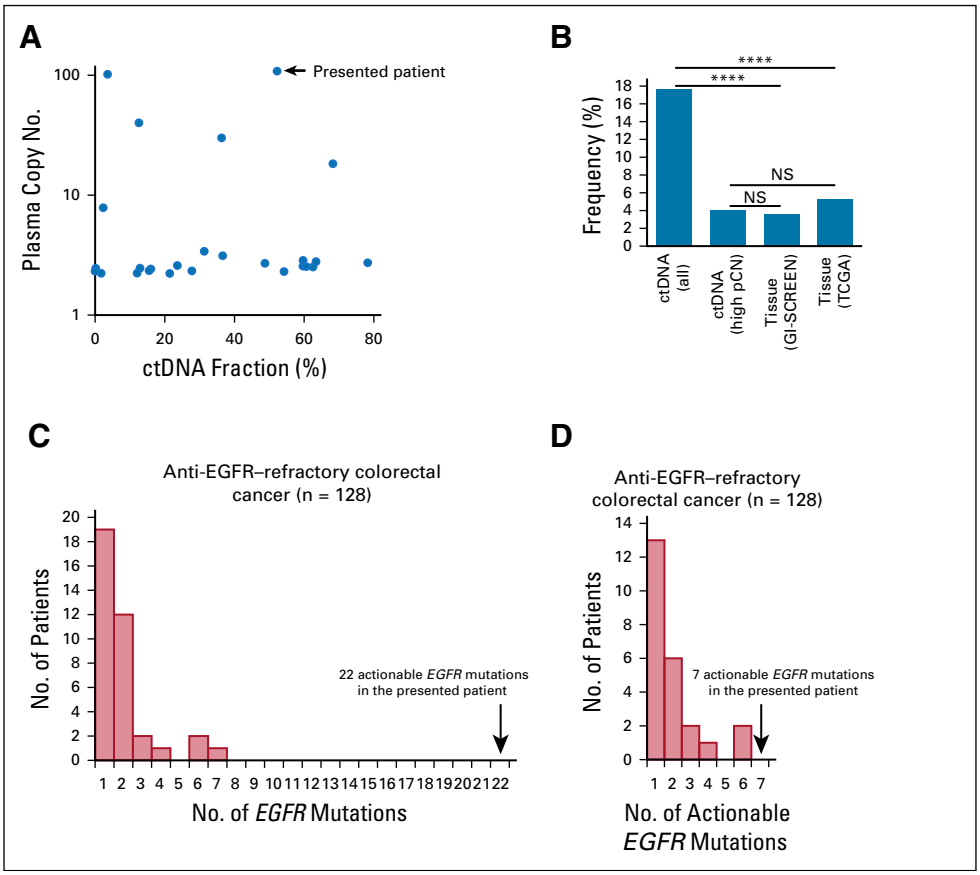

FIG 3. Genomic characteristics of advanced gastric cancer (GC) with epidermal growth factor receptor (*EGFR*) amplification in circulating tumor DNA (ctDNA). (A) Plasma copy number (pCN) versus ctDNA fraction as the maximum observed variant allelic frequency. (B) Frequency of *EGFR*-amplified GC in ctDNA versus GI-SCREEN and The Cancer Genome Atlas database. For the ctDNA population, the frequency of all *EGFR* amplifications and high *EGFR* pCN in GC is shown, respectively. (C) Distribution of the number of ctDNA *EGFR* mutations in patients with metastatic colorectal cancer after anti-EGFR therapy. (D) Distribution of the number of ctDNA actionable *EGFR* mutations in patients with metastatic colorectal cancer after anti-EGFR therapy. (****) $P <$ .0001. NS, not significant; TCGA, The Cancer Genome Atlas.

afatinib-containing treatment despite *EGFR* tyrosine kinase domain mutation in this patient may be associated with the multiple *EGFR* ECD mutations, for which the efficacy of afatinib has not been established. Gene amplification has been known to increase the likelihood of new gene mutations and then enhance the growth of subclones harboring a beneficial mutation.[20,21] The low variant allelic fractions of acquired *EGFR* mutations support the hypothesis that subclones with *EGFR* mutations that occurred in a part of amplified *EGFR* genes were increased by therapeutic pressure of anti-EGFR therapy. In addition to the heterogeneous and aggressive nature of GC, the remarkably highly amplified *EGFR* might cause far greater increase of the number of *EGFR* mutations than seen in mCRC and lead to short duration of response of cetuximab. *MET* amplification may be associated with resistance to targeted therapies in GC that harbors amplifications of RTK genes.[15,22] Of note, the acquired alterations predominantly occurred in chromosome 7. Given the baseline high *EGFR* pCN, the high instability across chromosome 7 might be associated with the rapid acquisition of resistance in this patient.

The concurrent emergence of the large number of *EGFR* mutations and *MET* amplification in this patient and findings of a previous study reporting various types of acquired gene alterations after anti-EGFR therapy,[15] indicate that the heterogeneity of *EGFR*-amplified GC is a great barrier for accurate therapy and warrants a novel strategy to overcome the heterogeneous resistance. Targeting heterogeneous secondary resistance alterations poses a clinical challenge because the majority of emerging mutations are not therapeutically actionable. Several strategies, such as antibody mixture, anti-EGFR combination, and antibody–drug conjugate, have been attempted.[23-25]

In conclusion, to our knowledge, this is the first report on the occurrence of multiple *EGFR* ECD mutations as a resistance mechanism to anti-EGFR therapy for *EGFR*-amplified GC. The use of ctDNA sequencing to identify *EGFR*-amplified GC and explore the resistance mechanism to anti-EGFR therapy requires additional evaluation to develop effective therapeutic strategies.

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