Int J Clin Exp Pathol 2019;12(3):1101-1107
www.ijcep.com /ISSN:1936-2625/IJCEP0089797

Original Article

Relationship between CpG island methylation phenotype, microsatellite instability phenotype and mutation of KRAS, NRAS, and BRAF genes in colorectal cancer

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Received December 13, 2018; Accepted February 13, 2019; Epub March 1, 2019; Published March 15, 2019

Abstract: Objective: CpG island methylation phenotype (CIMP) and microsatellite instability (MSI) are two different molecular mechanisms in colorectal cancer (CRC). Proto-oncogene KRAS, mutations in NRAS and BRAF play an important role in the formation of colorectal cancer. The correlation between the molecular typing of CIMP and MSI and the genes of KRAS, NRAS and BRAF was explored in this study. Methods: A total of 110 paraffin-embedded specimens of colorectal cancer were collected from the Chinese People's Liberation Army Rocket Army Special Medical Center during the period from May 2017 to September 2018. CIMP were detected by DNA methylation quantitative PCR (Methylight). Mutations in KRAS, NRAS, and BRAF genes were detected by realtime fluorescence quantitative PCR (qPCR); MSI typing was detected by sequencing. Results: Of the 110 colorectal cancer samples, 11 cases (10%) were CIMP-H, 92 cases (83.64%) were CIMP-L, and 7 cases (6.36%) were CIMP-0. 10 cases (9.09%) were MSI-H, and 100 cases (90.91%) were MSS and MSI-L. The mutation rates of KRAS, NRAS and BRAF genes were 50% (55 cases), 6.36% (7 cases) and 5.45% (6 cases), respectively. There was no significant correlation between CIMP group and MSI group ($P > 0.05$). Also, no significant differences were found in the mutations between the three subtypes of the CIMP group and the KRAS, NRAS genes ($P > 0.05$), while there was a statistically significant difference among the three subtypes of the BR and the BRAF gene mutations ($P < 0.0001$). There were no obvious differences between the three states of microsatellites and the mutations of KRAS and NRAS genes ($P > 0.05$), and the differences between them and BRAF gene mutations were statistically significant ($P < 0.01$). Conclusions: The BRAF gene mutation is closely related to the two types of CIMP and MSI, which may be an important part of the above two molecular mechanisms, and provide a reference for the treatment of the patients with CIMP-H and MSI-H.

Keywords: Microsatellite instability, CpG island methylation, BRAF

Introduction

The formation of colorectal cancer is thought to be primarily involved in three molecular pathway mechanisms [1]. The chromosomal instability (CIN) refers to chromosomal instability, such as the addition, deletion and translocation of chromosomes, accounting for about 85% of CRC; microsatellite instability (MSI) with mismatch repair gene deletion accounts for about 15%; and the phenotype of CpG island methylation is characterized by abnormal hypermethylation of CpG island in the promoter region of a specific gene and the silencing of tumor suppressor genes [2]. Mutations in KRAS, NRAS and BRAF genes are closely related to tumor differentiation, invasion and metastasis, and are important targets for clinical treatment of CRC. The aim of this study is to investigate the pathogenesis and relationship of two molecular types of CIMP and MSI under the influence of KRAS, NRAS and BRAF gene mutations [3].

Materials and methods

Specimen source

From May 2017 to September 2018, 110 cases of colorectal cancer paraffin specimens were removed by surgery or endoscopic biopsy in the Chinese People's Liberation Army Rocket Army

Special Medical Center. None of the patients received any radiotherapy, chemotherapy or molecular targeted drug therapy, and all of them were diagnosed and confirmed by more than two pathologists. Of the 110 samples, 70 (63.64%) were males and 40 (35.36%) were females, aged from 19 to 87 years, with a median of 66.

DNA extraction

We cut the paraffin wax block into sections and made sure that there was at least 50% of tumor tissue in each section. The thickness of each section was 5 μm. According to the tissue size, one block could be cut into 10 to 20 sections. The genomic DNA was extracted from paraffin specimens using the OMEGA FFPE DNA kit according to the instructions. Then, the DNA concentration with micro spectrophotometer SMA4000 made in Aide Biomedical Technology Co. Ltd. and the DNA extraction solution above 50 μg/μL was finally taken for the next experiment.

CIMP detection

Sodium bisulfite conversion: 350 μg of the above genomic DNA was taken, and the genomic DNA of the sample was modified and transformed by the EZ DNA Methylation-Gold TM KIT from ZYMO RESEARCH company according to the instructions.

Methylight detection of CIMP: CIMP determination index and PCR reaction primers and probe design [2] appeared in the References, in which primers and probes were synthesized by Beijing Tianyi Huiyuan Company, and the primer sequences are shown in **Table 1**. The Taqman probe reaction was carried out by using the 7500 Real Time PCR System of Applied Biosystems, and the reaction system was: Premix Ex TaqTM Hot Start (TaKaBa, Japan) 10 μL; modified DNA template 2 μL (10 μmol/L); The upper and lower primers 1.2 μL (10 μmol/L) respectively; the probe FAM 0.4 μL (10 μmol/L); the sterilized double distilled water 5.2 μL, a total of 20 μL. The reaction conditions were as follows: 50°C 2 min → 95°C 10 min → (95°C 15 sec → 60°C 1 min) × 40 cycles. One positive control group was set up for each PCR reaction, and the Sodium bisulfite Methylated Human DNA standard (ZYMO RESEARCH, USA) was used instead of the DNA template in the system, and a negative control group was set to replace the DNA template with sterile double distilled water.

Results interpretation: The CT value was less than 36 and the amplification curve appeared as the standard for methylation positive. The methylation index positive number ≥ 6 is CIMP-H; 0 < positive number ≤ 5 is CIMP-L; positive number = 0 is CIMP-0. We took the detection of CRABP1 gene methylation in two cases for an example (**Figure 1**).

MSI detection

According to the method in the literature [4], we extracted the genomic DNA of tumor tissue and adjacent normal tissues, and applied PCR reaction to six microsatellites of BAT-25, BAT-25, D5S346, D17S250, D2S12 and NR-24. The sites were amplified. The primer sequences are shown in **Table 1**. The reaction procedures were as follows: 92°C for 3 min → (95°C 30 s → 55°C 30 s → 72°C 30 s) × 30 cycles → 72°C 10 min → 4°C preservation. The reaction system was as follows: 6.25 μL of Premix Ex TaqTM Hot Start (TaKaBa, Japan), 4.25 μL of sterilized double distilled water, 1 μL of DNA template, and 0.5 μL of per upstream or downstream primer, for a total of 12.5 μL. The amplified product was sent to Tianyi Huiyuan Company for sequencing, and the result is shown in **Figure 2**. If there is only one site with replication misalignment, the tissue is considered to be microsatellite low instability (MSI-L); if there are two or more sites with replication errors, the tissue is considered to be highly unstable. (MSI-H); If there is no replication error at the above microsatellite sites, the tissue is considered to be Microsatellite Stabilization (MSS).

Detection of KRAS and NRAS genes

The mutation detection of KRAS and NRAS genes was carried out by using the human KRAS/NRAS gene mutation detection kit of Aide Biomedical Technology Co. Ltd., according to the instructions.

Detection of BRAF gene

The human BRAF gene V600E mutation detection kit of Aide Biomedical Technology Co., Ltd. was used for detection according to the product manual.

Table 1. Tested related primer sequences

Gene		CIMP primer
CACNA1G	Forward	TTTTTTCGTTTCGCGTTTAGGT
	Reverse	CTCGAACGACTTCGCCG
	Probe	6FAM-AAATAACGCCGAATCCGACAACCGA-TAMRA
IGF2	Forward	GAGCGGTTTCGGTGTCGTTA
	Reverse	CCAACTCGATTTAAACCGACG
	Probe	6FAM-CCCTCTACCGTCGCGAACCCGA-TAMRA
NEUROG1	Forward	CGTGTAGCGTTCGGGTATTTGTA
	Reverse	CGATAATTACGAACACACTCCGAAT
	Probe	6FAM-CGATAACGACCTCCCGCGAACATAAA-TAMRA
RUNX3	Forward	CGTTCGATGGTGGACGTGT
	Reverse	GACGAACAACGTCTTATTACAACGC
	Probe	6FAM-CGCACGAACTCGCCTACGTAATCCG-TAMRA
SOCS1	Forward	GCGTCGAGTTCGTGGGTATTT
	Reverse	CCGAAACCATCTTCACGCTAA
	Probe	6FAM-ACAATTCCGCTAACGACTATCGCGCA-TAMRA
CDKN2A	Forward	TGGAGTTTTCGGTTGATTGGTT
	Reverse	AACAACGCCCGCACCTCCT
	Probe	6FAM-ACCCGACCCCGAACCGCG-TAMRA
CRABP1	Forward	TCGAAATTTTCGTTGTTGCGT
	Reverse	TATCCGTACCTACCGCCGC
	Probe	6FAM-ACCATACCCAACTTCGCCGACACCTAA-TAMRA
MLH1	Forward	CGTTATATATCGTTCGTAGTATTCGTGTTT
	Reverse	CTATCGCCGCCTCATCGT
	Probe	6FAM-CGCGACGTCAAACGCCACTACG-TAMRA
COL2A1	Forward	TCTAACAATTATAAACTCCAACCACCAA
	Reverse	GGGAAGATGGGATAGAAGGGAATAT
	Probe	6FAM-CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA-TAMRA
ACTB	Forward	TGGTCATCCAGGTTTAGTAACT
	Reverse	AACCAATAAACCTACTCCTCCCTTAA
	Probe	FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA
MSI site		MSI primer
BAT-25	Forward	TCGCCTCCAAGAATGTAAGT
	Reverse	TCTGCATTTTAACTATGGCTC
BAT-26	Forward	TGACTACTTTTGACTTCAGCC
	Reverse	AACCATTCAACATTTTTAACCC
D5S346	Forward	ACTCACTCTAGTGAAATCGGG
	Reverse	AGCAGATAAGACAGTATTACTAGTT
D17S250	Forward	GGAAGAATCAAATAGACAAT
	Reverse	GCTGGCCATATATATATTTAAACC
NR-24	Forward	CCATTGCTGAATTTTTACCTC
	Reverse	ATTGTGCCATTGCATTCCAA
D2S123	Forward	AAACAGGATGCCTGCCTTTA
	Reverse	GGACTTTCCACCTATGGGAC

Statistical methods

Data analysis application SPSS 23. 0 statistical software, χ^2 and Fisher exact probability test, $P < 0.05$ indicated that the difference was statistically significant.

Results

The relationship between CIMP and MSI phenotype

Of the 110 colorectal cancer samples, 11 (10 %) were CIMP-H, 92 (83.64%) were CIMP-L, and 7 (6.36%) were CIMP-0. According to the MSI phenotype, 10 (9.09 %) MSI-H; 99 (90%) MSS; 1 MSI-L (0.91%). There was no significant difference between the CIMP groups and the MSI phenotype ($P > 0.05$) (**Table 2**).

Relationship between CIMP and KRAS, NRAS and BRAF genes

In 110 cases of colorectal cancer, there were 55 cases of KRAS mutations, of which 5 cases were CIMP-H; 55 cases of KRAS wild type, and 6 cases of CIMP-H. There was no significant difference between the groups ($P > 0.05$). NRAS mutations were detected in 7 cases, of which no CIMP-H; 11 cases of CIPS-H in NRAS wild type were found and the difference between the groups was not statistically significant ($P > 0.05$). There were 6 cases of BRAF mutations, including 4 cases of CIMP-H, and there were 104 cases of BRAF wild type, of which 7 cases were CIMP-H. The difference between groups was statistically significant ($P < 0.00001$) (**Table 3**).

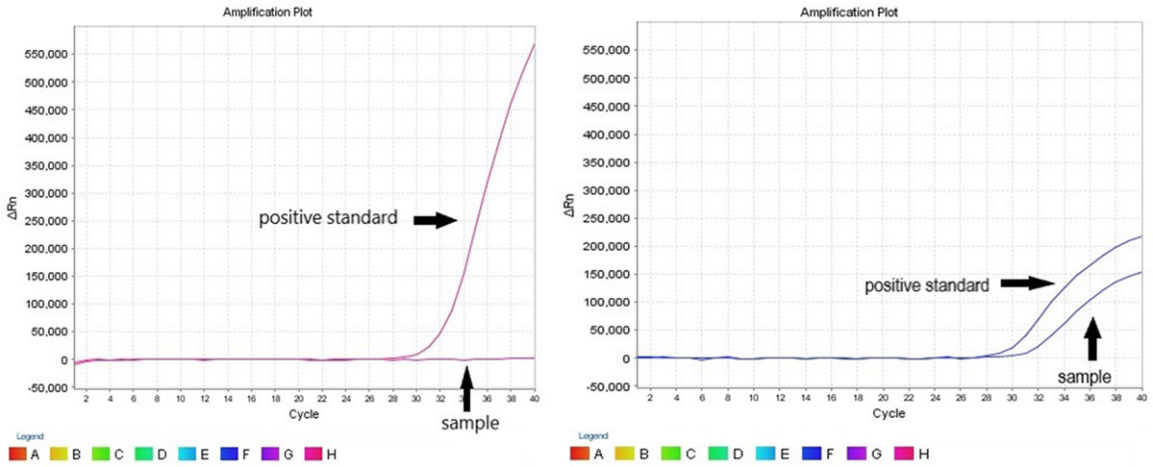

Figure 1. Left is negative for CRABP1 methylation and positive for right.

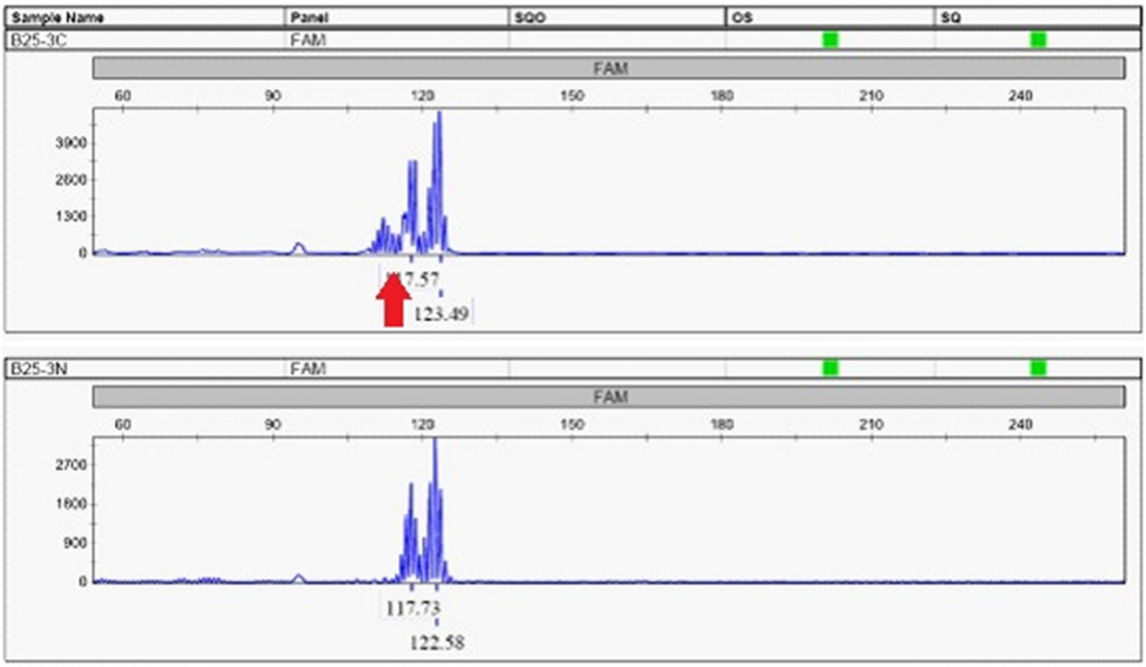

Figure 2. The arrow indicates a replication error in the tumor tissue relative to normal tissue, suggesting that this site is unstable.

Table 2. CIMP's relationship with MSI phenotype

CIMP	*N*	MSI			χ^2	*P*
		MSI-H	MSI-L	MSS		
CIMP-H	11	2	0	9	2.008	0.734
CIMP-L	92	8	1	83		
CIMP-0	7	0	0	7		

Use R × C contingency table χ^2 test.

Among the 110 cases of colorectal cancer, there were 3 cases of KRAS mutation with MSI-H, 52 cases of KRAS mutation with MSI-L or MSS and 7 cases of KRAS wild type with MSI-H; There were also 48 cases of simultaneous MSI-L or MSS in wild-type KRAS, and no significant difference between MSI group and KRAS group ($P > 0.05$) was found. In this trial, there were no cases of NRAS gene mutations occurring simultaneously with MSI-H; NRAS mutations were associated with MSI-L or MSS in 7 cases; NRAS wild type was associated with MSI-H in 10 cases; There were 93 cases of NRAS wild type and microsatellite stable type,

Table 3. CIMP's relationship with KRAS, NRAS and BRAF

CIMP	*n*	NRAS		χ^2	*P*	KRAS		χ^2	*P*	BRAF		χ^2	*P*
		Mutation	Wild			Mutation	Wild			Mutation	Wild		
CIMP-H	11	0	11	1.463	0.481	5	6	1.420	0.492	4	7	22.642	< 0.00001
CIMP-L	92	7	85			45	47			2	90	11.733	0.0006139
CIMP-0	7	0	7			5	2			0	7	0.43129	0.5114

Use R × C contingency table χ^2 test.

Table 4. MSI's relationship with KRAS, NRAS and BRAF

MSI	*n*	NRAS		χ^2	*P*	KRAS		χ^2	*P*	BRAF		χ^2	*P*
		Mutation	Wild			Mutation	Wild			Mutation	Wild		
MSI-H	10	0	10	0.831	0.660	3	7	2.853	0.240	3	7	13.001	0.0003113
MSI-L	1	0	1			0	1			0	1	0.000	1
MSS	99	7	92			52	47			3	96	11.282	0.001

Use R × C contingency table χ^2 test.

and there was no significant difference between MSI group and NRAS group (P > 0.05). In 110 cases of colorectal cancer in this trial, BRAF gene mutation and MSI-H occurred simultaneously in 3 cases; BRAF gene mutation but no MSI-H was found in 7 cases; BRAF mutation type with MSS or MSS- was found in 3 cases; there were 98 cases of BRAF wild type and microsatellite stability, and the difference between the two groups was statistically significant ($P < 0.001$) (**Table 4**).

Discussions

Colorectal cancer is a cancer that is formed by many aspects. For example, the activation of proto-oncogenes, the inactivation of tumor suppressor genes and the inactivation of mismatch repair genes have led to genetic and epigenetic changes in normal intestinal tissues, which ultimately lead to the formation of tumors.

In 1999, Toyota et al. [5] proposed the concept of CpG island methylation phenotype, in which a high proportion of DNA methylation occurred in the CpG island promoter region of CRC genome, resulting in the inactivation of its gene function, which was a unique molecular pathogenetic mechanism. Although the research on CIMP is very deep, there still exists great controversy in the method of identifying this molecular typing. In this paper, the method of Nosho and, Shima [2] was selected according to the eight methylation indexes of CACNA1G, CDKN2A, CRABP1, MLH1, IGF2, NEUROG1, SOCS1 and RUNX3, and CIMP was divided into CIMP-H, CIMP-L, CIMP-0. The methylation index positive number ≥ 6 is CIMP-H; 0 < positive number ≤ 5 is CIMP-L; positive number = 0 is CIMP-0. Among them, CIMP-H type CRC has a hypermethylation of CpG island promoter which causes its gene function to be inactivated, thus resulting in the inactivation of tumor suppressor gene and causing colorectal cancer. CIMP-L may not have extensive promoter methylation, compared to CIMP-H. It has been reported that CIMP-L is associated with KRAS mutations and male susceptibility [6], but this correlation has not been confirmed in this trial. In addition, it has been reported in the literature that BRAF mutations are closely related to KRAS wild type and female susceptibility [7]. We suspect that the correlation between CIMP-L and KRAS mutations in other trials may be indirectly caused by the negative correlation between CIMP-L and BRAF mutation ($P < 0.001$). Although the pathogenesis of CIMP-L may be related to the promoter methylation of tumor suppressor genes, the mechanism remains to be further elucidated. CIMP-0 is a special phenotype in CIMP and is considered to be the best prognostic CIMP classification [8]. In this test only 6.36% was involved. The CILP-0 type CRC did not undergo methylation of the above 8 methylation indicators, and this type has not been shown to be associated with promoter methylation. The KRAS mutation rate of CIMP-0 was the highest among the three CIMP types (KRAS mutation rate: CIMP-H 36%; CIMP-L 48% CIMP-0 57%), but this difference was not statis-

tically significant. The pathogenesis of CIMP-0 is still unclear and remains to be further studied.

Microsatellite instability is another molecular typing of CRC. Microsatellite DNA is also known as a simple repeat sequence. Microsatellites are involved in the regulation of many genes and have the functions of promoting the stability of chromosome structure and regulating mitosis, *etc*. [8]. However, microsatellite sequences are the most prone to gene mismatches, so they are the most sensitive sequences for mismatch repair genes. The instability of microsatellite often results in the inactivation of mismatch repair (MMR) genes, which are mainly manifest as the functional abnormalities of genes, like hMSH3, hMSH6, hPMS2 and hMLH3. This state of MMR dysfunction may cause a range of genetic problems. For example, in the tumor tissues of patients with Lynch syndrome (LS), the microsatellite states of these tumor tissues are mostly highly unstable and the MMR protein A is missing [9]. It has been reported in the literature that MSI-H is associated with CIMP-H [10], and the main reason is the methylation of hMLH1. This conclusion has not been confirmed in this experiment. One of the important reasons for MSI-H is the methylation of the mismatch repair gene. The methylation of the hMLH1 promoter is dominant. The positive rate of hMLH1 methylation in this experiment is 35.45%. When the hMLH1 index alone was compared with MSI, the results were not statistically significant (X = 0.014488, P = 0.9042), probably because methylation index (PMR) value of some samples had not reached the level of complete inactivation of the mismatch repair gene [11]. Then there was no significant relevance, which could be determined by further testing.

KRAS and NRAS belong to the same RAS gene. In normal cells, RAS protein binds to GDP and is in an inactive state. When KRAS and NRAS genes are mutated, the GTPase activity of RAS protein is blocked, so that GTP binding RAS protein is not hydrolyzed. RAS-GTP continues to activate subsequent pathways to stimulate the continuous growth and differentiation of cells, ultimately leading to malignant transformation of cells [12]. In the RAS-RAF-MAPK pathway, RAS-GTP binds to BRAF, resulting in a change in the conformation of the BRAF gene [13]. This mechanism may inhibit the mutation of BRAF gene after KRAS mutation, thus showing the mutual exclusion between BRAF mutant and KRAS mutant. In this study, no relationship was found between KRAS, NRAS, CIMP and MSI phenotypes, but it has been reported in the literature that it is closely related to chromosomal instability of (CIN) colorectal cancer [14]. CIN may be an important manifestation of KRAS and NRAS mutations.

As mentioned above, BRAF is an important gene in the RAS-Raf-MAPK pathway. When RAS-GTP protein binds to BRAF, it causes a conformational change of BRAF, which activates Raf protein, which in turn phosphorylates MEK and ERK proteins, thereby affecting the changes in transcription level and regulating physiologic activities of cells. However, the mutations in the BRAF gene will increase the activity of the product by more than 10 times, over-activate the MAPK pathway, and cause cell dysfunction, thus causing tumor [14]. It has been reported in the literature that overexpression of the MAPK pathway increases the activity of methyltransferases, which leads to the methylation of genes [15]. There was a significant positive correlation between BRAF and CIMP-H in this experiment, which may be a pathogenesis of CIMP. It has been documented that the main reason for the MSI-H of the BRAF mutant is the methylation of the hMLH1 promoter [10]. However, in this experiment, there were only two cases of BRAF mutation with MSI-H, showing different results of the methylation of hMLH1, and the difference was not statistically significant when compared with MSI-H of BRAF wild type.

CIMP and MSI phenotypes are two important mechanisms for the formation of colorectal cancer, and are important indicators for clinical prediction, diagnosis and treatment. Different CIMP and MSI have different clinical and pathological manifestations, and there are corresponding guidelines for the prediction of drug use and survival. Therefore, it is extremely important and necessary to perform CIMP and MSI phenotype testing in patients with colorectal cancer. In the future, more attention should be paid to the molecular typing of malignant tumors, including colorectal cancer, and the formation mechanism of molecular typing should be further explored. The treatment prin-

ciples and methods should be refined to achieve better therapeutic effect and survival prognosis.

Disclosure of conflict of interest

None.

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