Iraqi Journal of Industrial Research, Vol. 10, No. 1 (2023)

Open Access

Iraqi Journal of Industrial Research (IJOIR)

Journal homepage: http://ijoir.gov.iq

Study of the Association of Vitamin D Receptor (VDR) Gene Polymorphism in Iraqi Patients with Inflammatory Bowel Disease

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Article information

Article history:
Received: May, 23, 2023
Accepted: June, 05, 2023
Available online: June, 14, 2023

Keywords:
Inflammatory bowel disease,
(VDR) gene,
FokI gene polymorphism

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DOI:
https://doi.org/10.53523/ijoirVol10I1ID344

Abstract

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder divided into two types, Ulcerative colitis (UC), and Crohn′s disease (CD). In total, 80 blood samples were collected for this study, 40 blood samples were collected from IBD and 40 blood samples were collected from healthy individuals as. Genomic DNA extracted from whole blood and (FokI) polymorphism was detected by polymerase chain reaction Restriction fragment length polymorphism (PCR- RFLP). The results of the statistical analysis showed high frequency and percentage in age group (< 30 years) was 16 (40%) with significant (P≤0.05) when compeered with control group. The frequency and percentage of body mass index (BMI) for the patient group was (27.91 ± 0.45). While BMI was (27.31 ± 0.54) for the control group with highly significant association (p<0.01) between Iraqi patients with IBD when compared with control group. (VDR- FokI) wild allele PCR products (without restriction site) have 265bp (F allele) and when digested and electrophoresis of the digestion products on agarose gel the resulted fragment size of 196bp, 265 bp and 69 bp (f allele). The genotype of FokI gene (TT/FF) was 14 (35%) (P-value=0.0039), TG / Ff 15(37.5%) (p= 0.0253), GG/ff 11(27.5%) (p=0.0039) in patient group which have a highly significant in frequency and percentage, as compared with control group. In conclusion, The *FokI* genes polymorphism was shown to be associated with an increased incidence of Iraqi patients with IBD when compeered with control group. The genetic factors of *FokI* gene polymorphism may have a role in inflammatory bowel disease.

1. Introduction

Inflammatory bowel disease (IBD), is a complex and long-lasting inflammatory disease of the gastrointestinal tract that affects both grown-ups and children, including ulcerative colitis and Crohn's disease that are distinguished by their site in the bowel wall, most frequently UC affects the rectum, but then it may spread into the sigmoid, Crohn disease (CD) results in existing ulceration of any part of the gastrointestinal tract (GI) [1,2]. IBD has been widen

all the way through the world, in evolving countries it has been categorized via the prevalence of the rate of ulcerative colitis in compared to Crohn's disease, however, in the latest years there have been reports of increased occurrence of Crohn's disease. The society, microbiological, immunological, genetic and environment perspectives might be considered as possibility risk factors for the disease but then again it's still unclear [3,4] .

Several genome-wide association studies (GWAS) have been performed in large datasets from IBD genetics groups around the world, identifying to date 231 independent SNPs within 200 loci across populations [5]. Many of these variants are likely to affect the amount that gene is expressed (gene regulation) rather than the sequence of the protein product. One of the key critical players in preserving intestinal mucosal barrier integrity is 1,25(OH)2D3 (calcitriol), the active metabolite of vitamin D3 which exerts its regulatory function by binding to the vitamin D receptor (VDR), a phosphoprotein, a member of a superfamily of nuclear hormone receptors [6].

This signalling has complex effect on the immune system with anti-inflammatory, immune-modelling, anti-mitotic, pro differentiating and pro-apoptotic impact. Binding of1,25(OH)2D3 to the VDR promotes VDR hetero dimerization with the retinoid X receptor and cooperative coupling to vitamin D responsive elements, thus controlling the transcription of target genes [6].

VDR is encoded by a gene of more than 100 kb located on chromosome 12q12–14. It has 8 coding and 6 untranslated exons spanning together approximately 75 kb, which exhibit a high degree of polymorphism. The most common SNPs studied regarding various inflammatory based diseases are *FokI, ApaI, BsmI* and *TaqI* RFLP polymorphisms [7].

This study aimed to determine genetic variation of *Fok1* gene in Patients with Inflammatory Bowel Disease (IBD) and healthy control, and investigate the possible related with risk factor of this disease Theoretical Part

2. Experimental Procedure

Eighty Subjects divided into two groups (40 controls, 40 patients) with bowel disease which divided into two groups 20 patients with signs and symptoms suggestive of Crohn’s disease diagnosed by laparoscopy, while the rest 20 patients with ulcerative colitis were examined and selected at the Gastroenterology and liver teaching hospital/ Baghdad from (October 2022 to December 2022). The patients with age range are between (17-65) years were (20 males and 20 females) diagnosed to have bowel disease with either Crohn’s disease or ulcerative colitis.

Forty healthy subjects (24 males and 16 females) comparable in age to patients are involved in the study as controls. Those who considered as control have normal bowel disease test; with age range between (17-65) years. All the information has been obtained from all patients by direct interview. The blood specimens were collected from venous from each subject's group, 5 ml of whole blood were placed in tube containing EDTA (Ethylene Diamine Tetra Acetic Acid).

Genetic Analysis: Total genomic DNA was extracted from blood samples using DNA extraction kit supplied by Genaid / Korea, Polymerase Chain Reaction (PCR): Primers sequences have been to amplify the *FokI* fragment (265 bp). Forward: 5’AGC TGG CCC TGG CAC TGA CTC TGC TCT -3', and Reverse: 5'-ATG GAA ACA CCT TGC TTC TCC CTC-3'

All primers have been assessed by (Blast n) program which is available at the National Centre Biotechnology Information (NCBI) and each primer has been prepared with deionized water to reach the final concentration 100 picoMols/µl. Stored at (-20 ℃) until use.

After preparing the optimal reaction volume in PCR tube, the mixture has been spanned down and then PCR tube is placed in the PCR thermo cycler, and the optimal amplification reactions started according to the program PCR thermic conditions forward and reverse primer sequences. PCR product was 265bp of *FokI* for 2:00 hours, which performed with the temperatures profile consists of an initiation denaturation at 95 ℃ for 5 minutes proceeded by 35x cycle program with denaturation at 95℃ for 1 minutes, annealing at 60.8℃ for 45seconds, elongation at 72℃ for 54 seconds and final lengthening at 72℃ for 5 minutes. The presence of PCR product examined by

electrophorese on 1.5% agarose gel in 1X TAE buffer; DNA band (740 bp), (265 bp) has been visualized by electrophoresis and captured by gel documentation system to the observed band.

Restriction Fragment Length Polymorphism (RFLP): The following PCR creation, *FokI* restriction enzymes (Thermo Scientific, USA) and enzyme buffer could be utilized for consumption of PCR products (RFLP) to detected *FokI* (rs10735810, SNP C> T, allele F/f).

FokI PCR result was consumed in two hours in 37°C, the electrophoresis of consumption results on 1.5% agarose gel which utilized in determination FokI polymorphisms. For FokI site, 265 bp and 169+96 bp categorize alleles C (F) and T (f), respectively. PC the restriction site of FokI enzyme is 5'…../GGATG/….3' (rs10735810).

Statistical Analysis: The statistical analysis system (SAS) (SAS, 2018) programs was used to detect the effect of difference factors in study parameters. T-test was used to significant compare between means. Chi-square test was used to significant compare between percentages. In all statistical analyses, a P value (0.05 and 0.01) was considered to be significant.

3. Results and Discussion

3.1. Results

In this study a total of 80 blood samples were collected from IBD patients and healthy controls, 40 case-control subjects were enrolled to investigate the association of *FokI* polymorphism of VDR with susceptibility to CD and UC in Iraqi population. Subjects were divided into two groups: first group (1): included 40 IBD patients 24 male (60%) and 16 females (40%) categorized to 28 ulcerative colitis (70%) and 12 Crohn disease (30%).Group (2): included 40 healthy control individual, among them 20 (50%) male and 20 (50%) female. The distribution of patient and control group according to age we find high frequency and percentage in age group (> 30 years) was 16 (40%) with highly significant (p< 0.01) when compeered with control group as shown in table (3).

The results of this study showed no significant differences for IBD when compeered with control group as illustrated in table (4).

Table (1). Distribution of sample study according to Age groups in patients and control.

Factor		Patients (No=40)	Control (No= 40)	P-value
Age group: No (%)	<30 yr.	16 (40%)	5 (12. 5%)	0.038 *
	30-40 yr.	13 (32.5%)	9 (22.5%)	0.902 NS
	>40 yr.	11 (27.5%)	26 (%65)	0.037 *
	P-value	0.437 NS	0.0076 **	---
* (P≤0.05) ** (P≤0.01). NS: Non-Significant				

Table (2). Distribution of sample study according to Location in patients and control.

Factor		Patients (No=40)	Control (No= 40)	P-value
Location: No (%)	Urban	35 (87.5%)	36 (90%)	1.00 NS
	Rural	5 (12.5%)	4 (10%)	1.00 NS
	P-value	0.0001 **	0.0001 **	---
** (P≤0.01).				

Table (3) provide the demographic characteristics of the participants in this research the frequency and percentage of body mass index (BMI) for the patient group was (27.91 ± 0.45). While BMI was (27.31 ± 0.54) for the control group with highly significant association (p<0.01) between Iraqi patients with IIBD when compeered with control group.

Table (3). Distribution of sample study according to BMI in patients and control.

Factor		Patients (No=40)	Control(No= 40)	P-value
BMI: No (%)	Underweight<17.5	6 (15%)	0 (0.00%)	0.217 NS
	Normal:17.5-22.9	14 (35%)	4 (10%)	0.082 NS
	Overweight:>23	20 (50%)	36 (90%)	0.0086 **
	P-value	0.0092 **	0.0001 **	---
** (P≤0.01)				

Table (4). Comparison between patients and control groups in Age and BMI.

Group	**Mean ± SE**	
	Age (year)	**BMI (kg/m^2)**
Patients	33.23 ±1.82	24.23 ±0.82
Control	42.74 ±1.64	27.91 ±0.63
T-test	4.883 **	2.062 **
P-value	0.0002	0.0006
**** (P≤0.01).**		

Table (5). Sex effect of serum Ca, D3, Hb, Blood sugar, CRP levels.

Parameters	Mean ± SE		P-value
	Male	Female	
Ca (**(**mg/dL)	9.98 ±0.84	12.19 ±0.81	0.0639 NS
D3 (ng/dL)	16.28 ±1.37	15.67 ±1.02	0.0739 NS
Hb (g/dL)	12.93 ±0.39	11.56 ±0.26	0.0359 *
Blood sugar (mg/dL)	95.62 ±3.55	93.84 ±4.53	0.258 NS
CRP (mg/L)	±	±	0.961 NS
* (P≤0.05), NS: Non-Significant			

3.1.1. Genetic Analysis

DNA Extraction and Amplification

This study focused on the relation between VDR gene polymorphism and the incidence of inflammatory bowel disease by investigating two polymorphisms of FokI (rs2228570; exon 2), and Bsm1 (rs1544410; intron 8) genes.

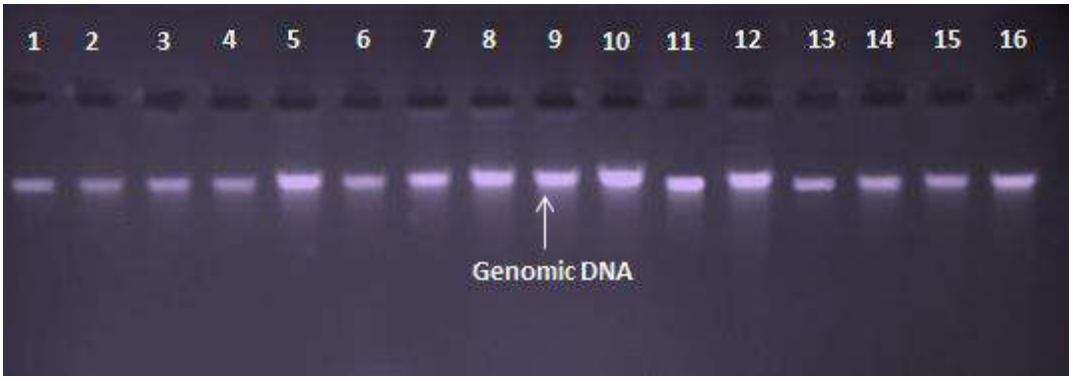

Figure (1). Electrophoresis of genomic DNA on agarose gel 1% at 90 volt for 30 min. Lane (1-8): IBD patients; Lane (9-16): healthy controls.

VDR) gene is amplified by PCR of (80) samples (40 patients and 40 healthy controls). Agarose gel electrophoresis of amplified PCR products show: The band of (*FokI*)) Polymorphic variant (VDR) gene at molecular weight (265 pb) and as shown figure (2).

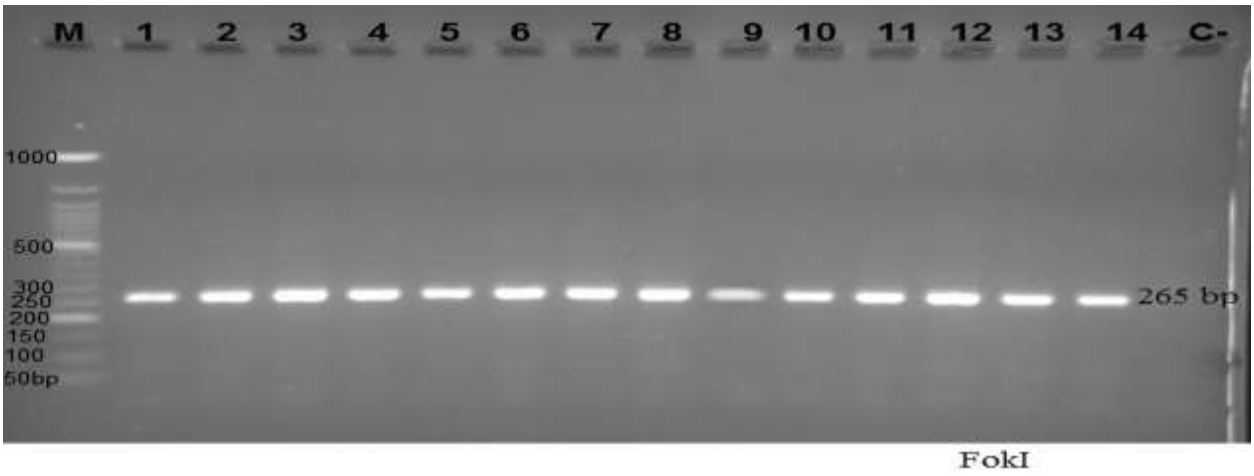

Figure (2). Gel electrophoresis of the PCR product (*FokI*) by (1.5%) (1h/90v). Lane M: DNA marker (50bp), Lane C-, negative control. Lanes (1-14) are samples.

Digestion of (VDR) Gene

Polymerase chain reaction products of (VDR- *FokI*) wild allele PCR products (without restriction site) have 265bp (F allele) and when digested for 3 hours in 55°C, and electrophoresis of the digestion products on 1.5% agarose gel the resulted fragment size of 196bp, 265 bp and 69 bp (f allele) , figure (3)

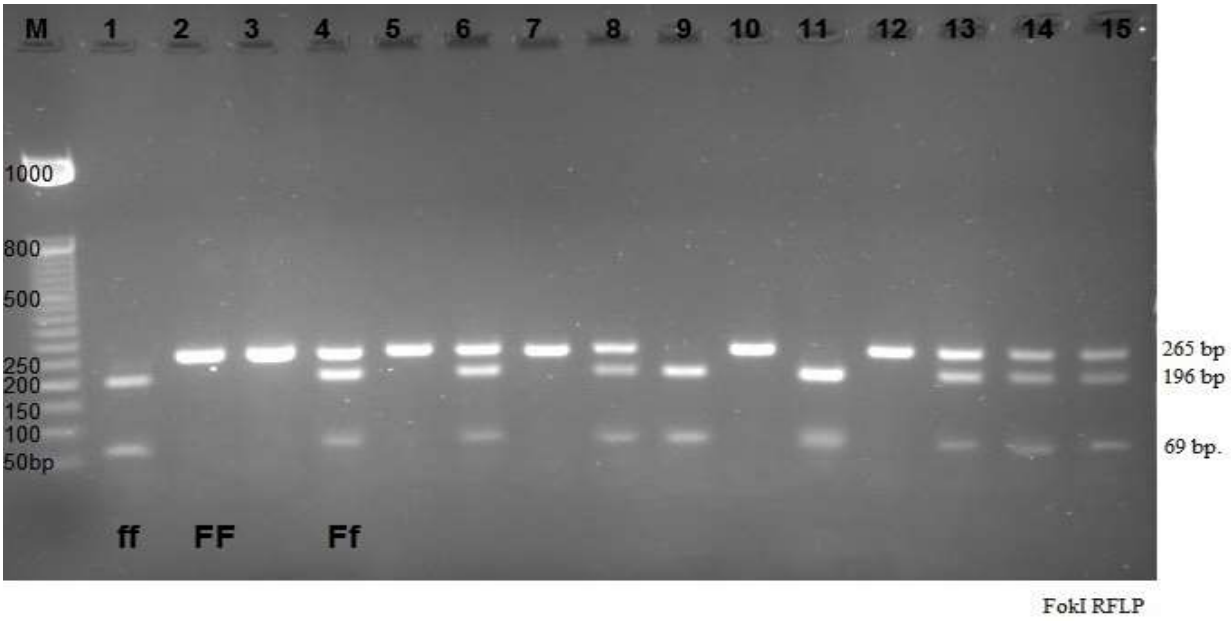

Figure (3). The PCR products of the (VDR) (*FokI)* gene after enzyme digestion and electrophoresis on 2% agarose gel. Show: Lane M : DNA marker (50bp), lane (4, 6, 8,13,14 ,15) heterozygous variant type Ff (265 bp and196bp, 69bp), lane (2, 3,7,10,12) homozygous wild type FF (265 bp) and lane (1,9,11) homozygous variant genotype ff (196bp,69bp).

The Association between genotype and allele of (*FOKI*) Polymorphism in VDR gene with IBD Patient and Control group: The study of the relationship between the study IBD Patient and control is shown in Table (8). The genotype (TT/FF) was 14 (35%), TG / Ff 15 (37.5%), GG /ff 11(27.5%) in patient group which have not significant (P>0.05) in frequency and percentage, as compared with control group.

The results of the multinomial regression in the table (6), showed that genotype TG/ Ff and GG/ff have (OR=0.372, 95%CI= 0.18-0.82, p-value=0.577) and (OR=0.502, 95%CI=0.26-1.07, p-value=0.206) respectively, with no

significant compared with control groups. Also data show the high prevalence and percentage of genotype in same group of Ff and ff (15 (37.50%), 11(27.50%) with highly significant ((P≤0.01). The Frequency of allele f in patients was record high percentage 30 (0.34) when compared with control groups 19 (0.22). Which indicated that allele G/f conceded risk factor of IBD.

Table (6). Genotype and allele frequency of FOK gene in patients and control groups.

Genotype/ *FokI* gene	Patients No. (%)	Control No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
TT /FF	14 (35%)	34 (85%)	1.042 NS	0.307	Ref. =1
TG /Ff	15(37.50%)	5(12.5%)	0.0310 **	0.577	0.372 (0.18-0.82)
GG /ff	11 (27.50%)	1 (2.5%)	0.0160 **	0.206	0.502 (0.26-1.-07)
Total	40	40	--		
P-value	0.0006 **	0.0001 **	--		
Allele	Frequency				
T/F	**56 (0.65)**	**67 (0.78)**	--		
G/f	30 (0.35)	19 (0.22)	--		
**** (P≤0.01), NS: Non-Significant.**					

These results of the current study demonstrated that there was significant differences in genotype distribution or in allelic frequencies between IBD patients and control group.

3.2. Discussion

The total number of patients (40) included (28) for the ulcerative colitis, (12) Crohn disease, while it was (40) for control group IBD (inflammatory bowel disease) is a multifactorial immunological deficiency characterized by persistent intestinal recurring inflammation of uncertain cause and characterized by a variable recurrence time and remission interval [8], IBD is linked to a number of complex elements as well. Environmental factors, metabolic flaws, and hereditary issues can all cause it. Body mass index (BMI) frequency and proportion, as well as participant demographics, were looked into. The results of the current study were consistent with Stephanie *et. al.* [9] study, which found that IBD prevalence is rising across the board, but is especially high among the elderly. According to this study, there are more cases of IBD in towns than in villages.

As compared to its rural population, the town diet contains significantly more inert inorganic non-nutrient micro particles like natural contaminants [soil and dust] and food additives, which may combine with intestinal luminal components like bacterial cell wall lipopolysaccharides to form antigenic particles. This suggests that the town diet may also affect the clinical course of IBD [8].

According to scientists [9] people with moderate disease activity had a minor prevalence of anemia, which is consistent with the association we found in this study between anemia and disease activity in IBD patients [10].The current study contradicts the study by Veli et al. [11], which demonstrated. Anemia occurred more frequently in IBD patients than in the control group. Hemoglobin 8.6 g/Dl was the incidence rate of anemia for the entire IBD. The current study is in line with a 2010 study by Bergamaschi et. al [12] that demonstrated anemia to be prevalent in IBD patients with active disease. IBD-related anemia appears to be complex. Additionally, IBD that is active and inflamed causes the inflammatory cytokines interleukin-6 and TNF-alpha, among others), which causes elevated hepcidin levels, it causes reduced release of iron from iron storing cells, leading to functional iron deficiency leading to anemia; also Higher oxidative stress may decrease erythrocyte life span [13].

Numerous studies have documented the connection between body mass index (BMI) and aspects of inflammatory bowel disorders (IBD), including morbidity, comorbidities, prognosis, the stage of the disease, and medical therapy. According to certain research, IBD patients' BMI was lower than that of their non-IBD controls. Others claimed that UC patients had BMIs that were normal or greater than those of controls. While some studies demonstrated that medical therapy could reduce lean mass, others showed that patients' BMI was lower than that of controls prior to medication. When compared to healthy controls, patients with CD did not frequently show lower BMI than those with active UC. However, several studies noted that individuals with inactive CD had lower BMIs than both UC and healthy controls [14].

The presence or lack of a restriction site for the enzymes *BsmI, ApaI,* and *TaqI* at the 3' untranslated region and *FokI* at the N-terminal region of the gene by RFLP is used to identify vitamin D receptor gene polymorphisms [15].

The starting codon and initiator zone of the VDR gene is where the *FokI* (rs2228570, at exon 2) is located [15]. It has been demonstrated that the FOKI polymorphism gene is linked to higher levels of physiological variables such calcium, D3, hemoglobin, blood sugar, and CRP. The active form of vitamin D in the human body is primarily in charge of preserving calcium homeostasis. Additionally, vitamin D3 stimulates the formation of collagen and proteins like osteopontin and osteocalcin that are essential for the mineralization of the bone matrix.

According to Uitterlinden *et al*. [16], the creation of a vitamin D-vitamin D receptor complex causes the activation or inhibition of target gene expression, which controls the synthesis of proteins involved in bone metabolism and maintains calcium homeostasis. Any modifications to the VDR gene may have an impact on the structural, functional, and/or activity of the VDR protein [17]. This might happen, for instance, by changing the transcriptional factor that is involved in the signal transduction from vitamin D to the genes that it regulates. Since they produce population-dependent, erratic results, VDR gene polymorphisms (*FokI, ApaI*) have attracted the attention of research teams all over the world for a long time. *FokI* VDR polymorphisms and poor bone mass in Japanese women have been linked in several studies, according to Pluskiewicz *et al.* [18]

Our results corresponded with previous study of Naderi *et al*., [19] so that showed frequency of the *FokI* polymorphism was considerably greater in the ulcerative colitis and Crohn's groups in the analysis of 230 Iranian people, including 150 patients with ulcerative colitis and 80 patients with Crohn's disease.

Vitamin D receptor (*BsmI, ApaI, and TaqI*) mutations and reduced 25(OH)D levels have been linked to CD in Chinese patients, according to the study by xia et al. [20]. The new findings suggested that inflammatory bowel illness may be caused by genetic variations in the *FokI* gene because of increased Frequency of allele f.

4. Conclusions

In conclusions, the finding of the present study suggested that *FokI* gene polymorphism is associated with inflammatory bowel disease. The association was found between *FokI* gene polymorphism with age and BMI factors in inflammatory bowel disease, also this study indicated to the significance association of inflammatory bowel disease in the urban with, the high frequency polymorphism of *FokI* gene.

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