LGALS3 and *AXIN1* gene variants playing role in the Wnt/β-catenin signaling pathway are associated with mucinous component and tumor size in colorectal cancer

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Abstract

The Wnt pathway alterations have been identified in colorectal and many other cancer types. It has been reported that galectin-3 (which is encoded by the *LGALS3* gene) alters the signaling mechanism in the Wnt/β-catenin pathway by binding to β-catenin in colon and other cancers. *AXIN1* is mainly responsible for the assembly of the β-catenin destruction complex in the Wnt pathway. This study investigated the relationship of rs4644 and rs4652 variants of the *LGALS3* gene and rs214250 variants of the *AXIN1* gene to histopathological and clinical properties. Our study included a total of 236 patients, of whom 119 had colorectal cancer (42 women, 77 men) and 117 were healthy controls. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and allele-specific oligonucleotide (ASO) PCR methods were used. In addition, the serum galectin-3 level was studied with the enzyme-linked immunosorbent assay (ELISA) method. For the rs4644 variant of the *LGALS3* gene, the CC genotype a mucinous component was significantly more common than those without a mucinous component (p=0.026). C allele frequency of the rs214250 variant of the *AXIN1* gene was significantly correlated to tumor size in the advanced tumor stage (p=0.022). The CCAACT haplotype was more common in colorectal cancer patients (p=0.022). Serum galectin-3 level was higher in the patient group compared to the control group (5.9± 0.69 ng/ml vs. 0.79±0.01 ng/ml; p<0.001). In conclusion, variants of *LGALS3* and *AXIN1* genes affect tumor sizes and the mucinous component via Wnt/β-catenin pathway in the pathogenesis of colorectal cancer.

KEY WORDS: Colorectal cancer; *LGALS3*; *AXIN1*; ASO-PCR, PCR-RFLP
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INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent cancer in both sexes worldwide. It is the second most common cause of cancer-related mortality in both sexes [1]. It is responsible for 9.4% of newly diagnosed cancer cases in men and 10.1% in women [2]. About 70% of the CRC cases are sporadic, i.e. no familial or genetic contribution can be found [3]. Genetic alterations or genomic re-arrangements can lead to important changes in cells with respect to both their function and the regulation of their proliferation [4]. Genetic derangements include activation of oncogenes (e.g. *K-ras*); inactivation of tumor suppressor genes (e.g. *APC, p53*) and DNA mismatch repair genes (e.g. *hMSH2, hMLH1*); and expression of genes (e.g. *COX-2*) with an unclear role in tumor formation, but which are considered to take part in the progression of tumor sequence [5]. A thorough understanding of colorectal carcinogenesis requires the elucidation of genetic mechanisms responsible for tumor development.

Galectin-3 is the only chimera galectin in vertebrates, and it has been widely studied. As a 29–35 kDa protein, it is involved in a series of processes in the human body, including cell adhesion, cell activation and chemoattraction, cell growth and differentiation, cell cycle, and apoptosis. Galectins are usually devoid of a signal peptide for involvement in the classical secretory pathway. However, galectin-3 has been isolated from

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extracellular space where it binds to a large number of molecules, mainly polylactosamine-rich molecules found in the extracellular matrix (ECM) or on cell surface. Galectin-3 has a vital function in the modulation of tumor progression in extracellular space [6-8]. It has recently been reported that it is also involved in the Wnt pathway and serves by binding to and promoting beta-catenin translocation into the nucleus [9]. It has also recently been demonstrated that the galectin-3 level is proportional to beta-catenin expression and the amount in colon cancer cells [9,10].

Iacovazzi et al. showed that galectin-3 levels were higher than the control group in well-differentiated colorectal cancers [11]. Immunohistochemical studies have demonstrated that galectin-3 is present in higher amounts in colorectal tissues of metastatic or recurrent cases [12]. Zaia et al. showed that it affects tumor-related survival by inhibiting apoptosis through binding to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Mazurek et al. reported findings supporting this information [13]. Animal studies have similarly shown higher galectin-3 expression [14]. Gene expression studies have indicated that the loss of galectin-3 expression is due to epigenetic factors in mucinous colorectal cases. Mahmoud et al. demonstrated that the DNA methylation profile of an *LGALS3* promoter was unmethylated in nonmucinous colorectal carcinomas, whereas *LGALS3* was aberrantly methylated in mucinous colorectal carcinomas. This has been regarded as important information pointing to differences in the pathogenesis of mucinous-type colorectal cancers [15].

Greco et al. demonstrated that the galectin-3 level was high in adenoma-containing tissues and blood samples of patients with adenomas [16]. Despite being a protein with well-defined functions in blood and tissue, no study has yet explored *LGALS3* (galectin-3) gene variations and mutations in colorectal cancers.

About 90% of colorectal cancers are characterized by some somatic mutations in genes taking part in the canonical, or β-catenin-dependent, Wnt pathway [17]. Although APC is the most commonly mutated Wnt pathway tumor suppressor gene, some CRCs and certain other malignancies have been found to contain germline or somatic mutations in the *AXIN1* or *AXIN2* genes [18]. *AXIN1* is mainly responsible for the assembly of the β-catenin destruction complex in the Wnt pathway, which serves as an inhibitory step in the expression of Wnt- and β-catenin-dependent target genes [19]. Alterations of the Wnt pathway are reportedly common in colorectal cancer with microsatellite instability, and *AXIN1* mutations have identified *AXIN1* as one of the genes that plays a role in these alterations [20]. In several studies, elevated levels of nuclear β-catenin in various human cancers have been demonstrated as a hallmark of active WNT/β-catenin signaling, while mutations of *AXIN* gene were significantly less frequent [21].

A genotyping study by Khan et al. conducted in a Kashmiri population revealed a codon D726D (GAT>GAC) variant causing no amino acid change in the first exon of *AXIN1* in cases with colorectal carcinoma [22]. Jin et al. detected 3 silent mutations and 6 missense point mutations in colorectal carcinoma [23]. Formerly known as an *AXIN1* inter-actor in the c-Jun NH(2)-terminal kinase pathway, *MAP3K1* also takes part in the canonical Wnt signaling pathway, where it positively regulates the expression of Wnt target genes [24]. Although there are a number of studies that examined the somatic mutations and functions of the *AXIN1* gene, gene variants have not been studied in detail.

The aim of our study was to investigate the relationship between the gene variants of *AXIN1* (rs214250) and galectin-3 (rs4644 and rs4652) genes and histopathological factors, and to determine the effect of genotype and haplotype on serum galectin-3 levels.

MATERIALS AND METHODS

Patients

Our study included a total of 236 patients with histopathological and demographic data, of whom 119 (42 women and 77 men) had colorectal cancer and 117 were healthy controls. The mean age of the colorectal cancer patients and the control group were 60.98 ±13.3 and 56.2±11.35 years, respectively. The demographic data is shown on Table 1.

Genotyping

All patients gave written informed consent. Istanbul University Ethics Committee approved the study. The peripheral blood samples were drawn into ethylenediaminetetraacetic acid (EDTA)-containing glass tubes. The salting-out process was used to obtain DNA material from peripheral circulating lymphocytes. Afterwards, polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) methods were utilized to genotype rs4644 and rs4652 variants of the *LGALS3* gene. For genotyping the rs214250 variant of the

TABLE 1. Demographic and medical history information

Parameter	Controls (*n*=117)	Patients (*n*=119)
Female, *n* (%)	52 (44.4)	42 (35.3)
Male, *n* (%)	65 (55.6)	77 (64.7)
Average age	56.2±11.35	60.98±13.3
Median age	55.5±3.69	57.9±5.80
Family history of cancer		
Available (%)	0 (0)	5 (4.2)
Not available (%)	117 (100)	114 (95.8)
Consumption of Alcohol		
Positive (%)	0 (0)	4 (3.4)
Negative (%)	117 (100)	115 (96.6)
Smoking		
Positive (%)	0 (0)	9 (7.6)
Negative (%)	117 (100)	110 (92.4)

AXIN1 gene, the ASO (allele specific oligonucleotide)-PCR method was used. PCR amplification was achieved using the following primers: Forward (wild-type): 5'- GAA GAC GGC GAT CCA TCG -3,' Forward (mutant type): 5'- GAG GAC GGC GAT CCA TCA -3' and Reverse: 5'- GGA TGC TCT CAG GGT TCT- 3' for *AXIN1* rs214250. The primers used for *LGALS3* gene variants rs4644 and rs4652 were Forward: 5'- TTA TCC TGG ACA GGC ACC TC -3' and Reverse: 5'- AAG GAA TGC CAT CTC ACC AG -3.' For the PCR process of all variants, the PCR conditions were set as follows: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 10 min. PCR product was studied in agarose gel electrophoresis in 148 bp for *AXIN1* genotyping. The PCR product Style enzyme containing the *LGALS3* gene rs4644 variant was treated at 37°C for 3 hours. The enzymatic restriction yielded restriction products of 201 bp for CC genotype, 204 bp, 170 bp, 31 bp for CA genotype, and 170 bp, 31 bp for AA genotype. The PCR product containing *LGALS3* gene rs4652 variant was treated by BsaWI enzyme at 60°C for 3 hours. The enzymatic restriction yielded restriction products of 201 bp for CC genotype, 201 bp, 134 bp, 67 bp for CA genotype, and 134 bp, 67 bp for AA genotype. PCR-RFLP and ASO PCR conditions of analysis of *LGALS3* and *AXIN1* variants are shown in Table 2.

Elisa

The enzyme-linked immunosorbent assay (ELISA) method was used to measure the galectin-3 level from the whole blood samples. The 96-well microplates were read at 450nm wavelength using Vivid Vision Microplate Reader (ALKA Tecnologia, São Paulo, Brazil).

Statistical analysis

Statistical analyses of the study data were done with the SPSS 11.0 software (SPPS Inc., Chicago, IL, USA). The two groups were compared by Chi square test and Fisher test with respect to differences of genotype and allelic frequency. Student's t test was used to compare the demographic variables of the two groups. The inter-group differences of ELISA results were analyzed with Kruskal-Wallis Test.

RESULTS

The demographic data are presented on Table 1. Genotypes of all genes were determined by gel band patterns (Figures 1 and 2). The distribution of the genotypes pertaining to the rs214250 variant of the *AXIN1* gene, the rs4644 variant of the *LGALS3* gene, and the rs4652 variant of the *LGALS3* gene were shown in Table 3.

No significant differences were found between the colorectal cancer and control groups with regard to genotype distribution and allelic frequencies for the rs214250 variant of the *AXIN1* gene (p>0.05). In contrast, the C allele was significantly more common (92.6%, 63 patients) in the advanced tumor stage (T3+T4) than in the early tumor stage (73.7%, 14 patients) in the rs214250 variant of the *AXIN1* gene (p=0.022, OR: 1.257 (0.953-1.659)).

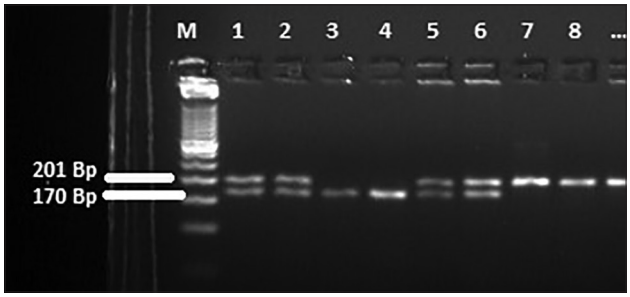

FIGURE 1. PCR-RFLP agarose gel electrophoresis of Rs4644 variant of ***LGALS3*** gene. M: marker; Lane 7 and 8: CC genotype (201 bp); Lane 1,2,5 and 6: CA genotype (201 bp and 170 bp). Lane 3 and 4: AA genotype (170 bp).

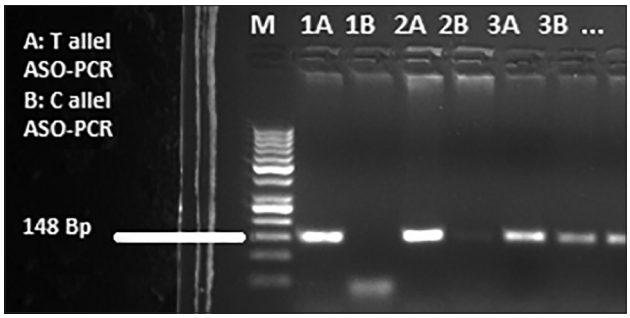

FIGURE 2. ASO-PCR agarose gel electrophoresis of Rs214250 variant of ***AXIN1*** gene. M: marker; C allele: 1A, 2A, 3A; T allele: 1B, 2B, 3B in patients and control groups.

TABLE 2. PCR-RFLP and ASO-PCR conditions of *LGALS3* and *AXIN1* variants analysis

Gene	Primer (forward)	Primer (reverse)	Annealing temperature	PCR product size	RFLP Enzyme	PCR-RFLP (fragment size)
LGALS3 (Rs4644)	5'- TTA TCC TGG ACA GGC ACC TC -3'	5'- AAG GAA TGC CAT CTC ACC AG -3'	60°C	201 bp	StyI	201 bp, 170 bp, 31 bp
LGALS3 (Rs4652)	5'- TTA TCC TGG ACA GGC ACC TC -3'	5'- AAG GAA TGC CAT CTC ACC AG -3'	60°C	201 bp	BsaWI	134 bp, 67 bp
Gene	Primer (common)	Primer (wild type)	Primer (mutant type)	PCR product size	Annealing temperature (°C)	
AXIN1 (Rs214250)	5'- GGA TGC TCT CAG GGT TCT- 3'	5' – GAA GAC GGC GAT CCA TCG – 3'	5'- GAG GAC GGC GAT CCA TCA -3'	148 bp	60°C	

Genotypes of the rs4644 variant of the *LGALS3* gene had a significantly different distribution between patient and control groups (p=0.0260). For the rs4644 variant of the *LGALS3* gene, the CC genotype carrying a mucinous component was significantly more common than those without a mucinous component (87.5% vs. 44.3%; p=0.026, OR: 1.975 [1.387-2.831]). Histopathologic criteria are presented in Table 4. Genotypes of the rs4652 variant of the *LGALS3* gene had a significantly different distribution between patient and control groups. In contrast, the genotype distributions were not significantly correlated to clinical and pathological parameters (p=0.0390).

TABLE 3. The distribution of Axin rs214250, Gal3 rs4644 and Gal3 rs4652 alleles in patients and controls

Genotypes and alleles	Patients *n*(%)	Controls *n*(%)	*p*-value
Axin rs214250			
CC	74 (62.2)	77 (65.8)	0.260
CT	33 (27.7)	23 (19.7)	
TT	12 (10.1)	17 (14.5)	
C allele	181 (76.1)	177 (75.6)	0.917
T allele	57 (23.9)	57 (24.4)	
Gal3 rs4644			
CC	62 (52.1)	61 (52.1)	0.026
CA	52 (43.7)	40 (34.2)	
AA	5 (4.2)	16 (13.7)	
C allele	176 (74)	162 (69.2)	0.25
A allele	62 (26)	72 (30.8)	
Gal3 rs4652			
AA	46 (38.7)	48 (41)	0.039
AC	65 (54.6)	50 (42.7)	
CC	8 (6.7)	19 (16.2)	
A allele	157 (66)	146 (62.4)	0.418
C allele	81 (34)	88 (37.6)	

The AACC haplotype of the rs4652 variant of the *LGALS3* gene and the rs214250 variant of the *AXIN1* gene had a significantly lower frequency in the patient group compared to the control group (3.4% vs. 13.7%, p=0.004; OR: 0.246; 95%CI: 0.085-0.713).

The CCCT haplotype of the rs4644 variant of the *LGALS3* gene and the rs214250 variant of the *AXIN1* gene had a significantly higher frequency in the patient group compared to the control group (18.5% vs 9.4%, p=0.044; OR: 1.966; 95%CI: 0.999-3.871).

In triple haplotyping of the rs4652 and rs4644 variants of the *LGALS3* gene and the rs214250 variant of the *AXIN1*, the CCAACT haplotype was significantly more common in the patient group than in the control group (12.6% vs. 4.3%, p=0.022; OR: 2.95; 95%CI: 1.108- 7.855).

The serum galectin-3 level was significantly higher in the patient group than in the control group (5.9±0.69ng/ml vs. 0.79±0.01ng/ml, p<0.001).

DISCUSSION

Colorectal cancer, the third most prevalent cancer worldwide, has become a major public health problem with an excess of 1.2 million new cases each year [26]. In the majority of CRC cases, there is no identifiable family history or genetic

TABLE 4. Clinical and histopathological data of colorectal cancer patients

Histopathological criteria	Galektin3 rs: 4644, *n* (%)			Galektin3 rs: 4652, *n* (%)			*AXIN1*, *n* (%)		
	CC	CA	AA	AA	AC	CC	CC	CT	TT
Gender									
Female	21 (50)	20 (47.6)	1 (2.4)	15 (35.7)	27 (64.3)	0 (0)	23 (54.8)	15 (35.7)	4 (9.5)
Male	41 (53.2)	32 (41.6)	4 (5.2)	31 (40.3)	38 (49.4)	8 (10.6)	51 (66.2)	18 (23.4)	8 (10.4)
T Stage									
T3+T4	32 (47.1)	34 (50)	2 (2.9)	25 (36.8)	38 (55.9)	5 (7.4)	46 (67.6)	17 (25)	5 (7.4)
T1+T2	10 (52.6)	7 (36.8)	2 (10.5)	7 (36.8)	10 (52.6)	2 (10.5)	9 (42.4)	5 (26.3)	5 (26.3)
N stage									
N1+N2+N3	23 (52.3)	18 (40.9)	3 (6.8)	18 (40.9)	22 (50)	4 (9.1)	27 (61.4)	11 (25)	6 (13.6)
N0	19 (44.2)	23 (53.5)	1 (2.3)	14 (32.6)	26 (3.7)	3 (7)	28 (65.1)	11 (25.6)	4 (9.3)
M Stage									
M1	10 (55.6)	7 (38.9)	1 (5.6)	7 (38.9)	10 (55.6)	1 (5.6)	9850)	6 (33.3)	3 (16.7)
M0	32 (45.7)	35 (50)	3 (4.3)	25 (35.7)	39 (55.7)	6 (8.6)	47 (67.1)	16 (22.9)	7 (10)
Angio-lymphatic invasion									
Positive	15 (55.6)	11 (40.7)	1 (3.7)	9 (33.3)	16 (59.3)	2 (7.4)	14 (51.9)	8 (29.6)	5 (18.5)
Negative	25 (43.9)	29 (50.9)	3 (5.3)	21 (36.8)	31 (54.4)	5 (8.8)	38 (66.7)	14 (24.6)	5 (8.8)
Perineural invasion									
Positive	11 (37.9)	17 (58.6)	1 (3.4)	8 (27.6)	20 (69)	1 (3.4)	19 (65.5)	8 (27.6)	2 (6.9)
Negative	30 (52.6)	24 (42.1)	3 (5.3)	23 (40.4)	28 (49.1)	6 (10.5)	35 (61.4)	14 (24.6)	8 (14)
Differentiation									
Low	6 (66.7)	3 (33.3)	0 (0)	5 (55.6)	4 (44.4)	0 (0)	4 (44.4)	4 (44.4)	1 (11.1)
Medium-high	17 (45.9)	17 (45.9)	3 (8.1)	13 (35.1)	17 (45.9)	7 (18.9)	23 (62.2)	9 (24.3)	5 (13.5)
Mucinous component									
Mucinous Ca	7 (87.5)*	1 (12.5)	0 (0)	4 (50)	3 (37.5)	1 (12.5)	5 (62.5)	2 (25)	1 (12.5)
Non-Mucinous Ca	35 (44.3)	40 (50.6)	4 (5.1)	28 (35.4)	45 (57)	6 (7.6)	50 (63.3)	20 (25.3)	9 (11.4)

alterations, and the disease is largely sporadic. It has recently become clearer that CRC is a heterogeneous malignancy where prognosis and (targeted) treatment response in a given patient are largely dependent on molecular, as well as genetic properties, of the tumor [25, 26]. Unfortunately, there is still insufficient information about the molecular pathology of CRC.

The canonical Wnt pathway is responsible for the determination of cell fate at the developmental stages and in adult cellular homeostasis, and its dysregulation is responsible for a variety of malignant disorders [27]. The canonical Wnt pathway conveys extracellular Wnt signals into the cell nucleus by affecting levels and localization of β-catenin. The formation of β-catenin destruction complex involves the *AXIN1*, and *AXIN2* proteins [28]. Previous studies have shown that *LGALS3* plays a role in the Wnt/β-catenin pathway and is involved in cell migration and invasion, with its levels proportional to β-catenin in different cancer types [29,30].

In colorectal cancer cases, serum galectin-3 levels and immunohistochemical studies at tissue level have both supported the proposed pathogenesis [11,12]. Experimental animal models have similarly shown increased *LGALS3* m-RNA levels in colorectal tissue [14]. However, studies examining mutations and genetic variants have been limited in number so far. This creates an uncertainty surrounding the question as to whether the upregulation of protein level occurs somatically or as a result of germline gene mutations and/or gain-of-function mutations. Our findings showed that in the patient group with the rs4644 variant of the *LGALS3* gene, the number of patients with a CC genotype carrying a mucinous component was significantly greater than those with a CC genotype carrying a non-mucinous component (p=0.026). As for the rs4652 variant of the *LGALS3* gene, no significant differences were observed between the clinical parameters of the patient and the control groups. Mucinous adenocarcinoma (MUC) represents a histological subtype of CRC, containing a large amount of extracellular mucin in its structure. A large body of literature suggests that colorectal cases with a mucinous component carry a worse prognosis compared to those without such a component [31-33]. The mucinous component is more common in colorectal cancer cases that arise from inflammatory processes. The findings reported by Akkirpik et al. suggest that mucinous secretion of Kras mutations in the colo-rectum may represent a distinct genetic pattern [34]. Kawasaki et al. demonstrated the effect of methylations in the WRN promoter region on mucinous component development [35]. Its relationship with a mucinous component has been demonstrated in *SIRT1* histone deacetylase overexpression in colorectal cancer tissues [36].

The level of the interaction between epithelial cells without galectin-3 expression and the extracellular matrix is particularly low. Galectin-3 displays both pro- and anti-adhesive properties and functions as a matricellular protein in various types of the processes. Reduced cell-to-cell interaction and adhesion may contribute to the development of a mucinous component by supporting an inflammatory process. Genetic studies investigating the mucinous component are quite limited and the *LGALS3* rs4644 variant CC genotype was correlated to a mucinous component in our study. Cell culture-based studies are needed to explore the effect of galectin-3 (*LGALS3*) on the development of a mucinous component in colorectal cancers. *AXIN1* has traditionally been regarded as a tumor-suppressor protein. A majority of gastrointestinal cancer types carry *AXIN1* mutations [19]. Khan et al. [22] detected a mutation that caused no amino acid alteration, while Jin et al. [23] found 3 silent and 6 missense mutations in colorectal cancers. Despite a limited number of functional studies, Sue et al. reported an active role of *AXIN1* in the Wnt and c-Jun NH(2)-terminal kinase pathway [24]. Although there exist some data suggesting a role for engaging a cell into apoptosis, C allele frequency of the rs214250 variant of the *AXIN1* gene was significantly correlated to tumor size in the advanced tumor stage (T_3+T_4) (p=0.022). These findings may suggest that *AXIN1* plays a role, not only in apoptosis, but also in cancer cell growth and survival. The function of *AXIN1* in tumorigenesis should be studied in detail in animal model studies with *AXIN1* knock-out colorectal cancer and cell culture studies.

According to combined haplotyping results, the AACC haplotype was quite common in the healthy control group (p=0.004). CCAACT haplotype, on the other hand, was three times more common in colorectal cancer patients compared to the control group (p=0.022). These haplotypes can be used after cohort studies as a protective and prognostic biomarker in colorectal cancers. Despite the available data, the limitation of this study is the limited sample size. Future studies with a larger sample size may provide detailed information related to other pathological parameters.

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DECLARATION OF INTERESTS

The authors have declared that there is no conflict of interest.

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