symposium article

Gastric adenomas: relationship between clinicopathological findings, *Helicobacter pylori* infection, APC mutations and COX-2 expression

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Gastric adenomas are rare neoplastic growths characterized by localized polypoid proliferations of dysplastic epithelium that tend to progress to infiltrating adenocarcinoma. Therefore, the identification of molecular markers that could reliably recognize adenomas at risk of progression is advocated in the clinical management. In this study we investigated, in a series of gastric adenoma specimens from an area at high risk of gastric cancer, the relationship between clinicopathological characteristics of adenoma and *Helicobacter pylori* infection, APC mutational status, and COX-2 and the down-stream enzyme mPGES1 expression. *Helicobacter pylori* infection, detected in 24%, and 33% by histology and PCR analyses, respectively, did not show any relationship with growth pattern, localization, size, dysplasia grade and presence of synchronous cancer. Pathogenetic mutations of MCR region (codons 1269–1589) of the APC gene were detected only in one case corresponding to a single, small size, low grade, *H. pylori*-negative adenoma. The expression of COX-2 largely matched that of mPGES$_1$. Both were overexpressed in 79% of cases showing a relationship with high-grade dysplasia, size >10 mm and presence of a synchronous carcinoma. In conclusion, COX-2 may play a key role in the development and progression of gastric adenoma and could be an attractive target in the management of gastric adenoma at major risk of cancer development.

Key words: APC mutations, COX-2, gastric adenoma, *Helicobacter pylori*

introduction

Gastric adenomas are rare neoplastic growths characterized by localized polypoid proliferation of dysplastic epithelium that generally arise against a background of mucosal atrophy and intestinal metaplasia [1]. Gastric adenomas tend to progress to infiltrating adenocarcinoma and are associated with a higher risk of adenocarcinoma elsewhere in the stomach [2–5]. Tumor size and histological grading of dysplasia have been indicated as prognostic markers [6]. Adenomas with high-grade dysplasia progress to adenocarcinoma in the majority of cases while those with low-grade dysplasia may persist unchanged or in a small minority progress to cancer [7, 8]. The identification of molecular markers that could reliably recognize adenomas at risk of rapid progression may be helpful in the clinical management of these patients.

Somatic mutations in the APC gene that tend to target a gastric mutation cluster region (MCR) extending between codons 1269 and 1589, are implicated in the pathogenesis of fundic adenomas [9]. However, unlike APC mutations in the colorectum [10, 11], a negative relationship between APC gene mutations and the development of adenocarcinoma has been noted, which agrees with the observation that fundic adenomas of familial adenomatous polyposis (FAP) patients rarely progress to cancer [9].

Cyclooxygenase-2 (COX-2), the rate limiting enzyme in the conversion of arachidonic acid to prostaglandins, is implicated in the progression of colorectal adenomas and FAP [12, 13]. The biological effects of COX-2 are partly mediated through PGE$_2$, the product of microsomal prostaglandin (PG) E synthase 1 (mPGES$_1$) [14]. COX-2 and mPGES$_1$ expression is up-regulated in gastric carcinoma as well as in gastric precancerous lesions [15–19]. The expression of COX-2 in gastric mucosa seems to be up-regulated by *Helicobacter pylori* infection, a well known carcinogenetic factor according to Correa's model [15, 16].

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There are numerous data in the literature concerning the association between *H. pylori* and gastric cancer [20–24]. In contrast, the role of bacterium in the development and progression of gastric adenoma remains unclear.

In this study we investigated a series of gastric adenoma specimens from an area at high risk for gastric cancer in northern Italy. The aim was to analyse the relationship between clinicopathological characteristics of gastric adenomas and the presence of *H. pylori* infection, APC mutational status, and expression of COX-2 and the downstream enzyme mPGES$_1$.

patients and methods

pathological characterization

The study series consisted of 29 paraffin-embedded gastric adenoma cases. The specimens were obtained during gastrectomy performed for synchronous gastric cancer or by polypectomy performed during upper endoscopy. For each case, sections of tumoral and peritumoral areas were studied. The cases were grouped in three subsets according to the Vienna classification [7]: low-grade adenoma/dysplasia, high-grade adenoma/ dysplasia and intramucosal carcinoma. Briefly, adenomas with low-grade dysplasia showed elongated, hyperchromatic and crowded nuclei with mild pseudostratification. Cribriforming architecture, marked glandular crowding, full-thickness nuclear stratification, and /or severe cytologic atypia were criteria for high-grade dysplasia. The adjacent non-tumoral mucosa were evaluated after staining with hematoxylin and eosin, Giemsa (for identification of *H. pylori*) and PAS/Alcian blue at pH 2.5 (for evaluation of intestinal metaplasia). In non-adenomatous gastric mucosa the presence of *H. pylori*, grade of inflammation, glandular atrophy, and intestinal metaplasia were classified according to the up-dated Sydney system [25]. Finally, synchronous carcinomas were classified according to Lauren's criteria [26].

immunohistochemistry

For each biopsy, 4-μm thick serial sections were cut from paraffin blocks, mounted on acid-cleaned glass slides and heated at 55°C for 60 min. Slides were de-waxed and re-hydrated, then endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2 in methanol (20 min at room temperature). To reduce non-specific background staining, slides were incubated with 5% goat serum (15 min at room temperature). Finally, slides were incubated with the appropriate primary antisera in a moist chamber overnight at 4°C. Immunohistochemistry for COX-2 and mPGES$_1$ was performed using specific antibodies to COX-2 (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and mPGES$_1$ (dilution 1:800; Santa Cruz Biotechnology, Santa Cruz, CA). The avidin–biotin–peroxidase complex procedure (ABC standard, Vector Laboratories, Burlingame, CA, USA) was then performed. Peroxidase activity was detected with diaminobenzidine (DAB) as the substrate. Finally, sections were weakly counterstained with Harris's hematoxylin and mounted. Two independent approaches were used to confirm the specificity of the immunohistochemical signal: serial dilution of the primary antibody until the signal disappeared; and the use of non-immune rabbit IgG instead of primary antibody, which failed to reveal relevant staining.

The intensity of the staining of both COX-2 and mPGES$_1$ was estimated on a scale from 0 (absent) to 4 (strong) and the area of positivity was assessed by providing values of 1 (focal or <10%), 2 (10%–30%), 3 (30%–50%), or 4 (>50%). Scoring was independently performed by two observers (RC and AR) and in the case of any disagreement, consensus was reached by direct discussion.

DNA extraction

Paraffin-embedded sections were collected on microscope slides. Tumor and tumor-free mucosal areas were identified within 10 μm-thick deparaffinized sections lightly counterstained with hematoxylin and microdissected into 1.5 ml polypropylene vials, using a hematoxylin and eosin-stained step section from the same block as a guide. DNA extraction was performed as previously reported [27].

analysis of the APC gene

Mutations in the *APC* MCR (codons 1269 to 1589) were determined by PCR and direct sequencing. PCRs were performed by amplification of six partially overlapping segments, using a 'seminested' amplification protocol (primers available upon request). Sequencing was performed on an ABI 310 Genetic Analyzer (Applied Biosystem). Only 16 cases yielded DNA quantitatively and qualitatively sufficient for mutational analysis of the APC gene.

genetic analysis of *H. pylori*

The presence of the *glmM* gene was determined in DNA samples extracted from normal and adenoma areas by semi-nested PCR and sequence analysis. Unavailability of freshly-frozen specimens required *ad hoc* development of PCR-based assays specifically designed for short *H. pylori* sequences retrievable from paraffin-embedded samples. On the basis of sequence alignments performed using the CLUSTAL W software, we designed primer sets [F1 (5′-TAACCGAAGACATGCGCTG-3′) and F2 (5′-AGACATGCGCTGTGATGC-3′) for a 144 base-pairs (bp) fragment; R (5′-CATGAAAGATTTCTTCAATCAATCGCT-3′) for a 137 bp fragment] targeting a conserved region of the *glmM* (*ureC*) gene. PCRs (conditions available upon request) were performed in a GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystems, Foster City, CA, USA). Amplified fragments were visualized on a 3% agarose gel electrophoresis stained with ethidium bromide. The PCR products were directly sequenced using an ABI PRISM Big Dye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequence variants were always confirmed using independent DNA preparations.

statistical analysis

Data was analyzed using the SPSS package for Windows. The categorical variables were analyzed using the χ^2 test or Fisher's exact test. $P < 0.05$ was taken as level of significance.

results

The clinical characteristics of the 29 patients with adenomas are shown in Table 1. Patients in the series were more frequently males than females (69% versus 31%). The mean age was 68 years (range 50–82). Adenomas were prevalently flat and were roughly equally distributed (45% and 55% in the proximal and distal stomach, respectively). Size ranged from 3 to 15 mm with most cases <10 mm (79%). The grade of dysplasia, evaluated according to the Vienna classification, was high in 55% of the cases and low in the remaining 45%.

The histological characteristics of the gastric mucosa surrounding the adenomas in relation to the localization, growth pattern, size, dysplasia grading of the adenomas and presence of synchronous gastric cancer are reported in Table 2. Gastric activity was higher in flat adenomas <10 mm and with low-grade dysplasia than in polypoid adenomas >10 mm and with high-grade dysplasia. Atrophy and intestinal metaplasia were present in the mucosa adjacent to the adenomas in all cases in which the size of the lesion was >10 mm. No significant

difference was found between presence of atrophy and intestinal metaplasia regarding growth pattern, dysplasia grade and location of adenomas.

Helicobacter pylori status evaluated by histology and PCR analysis targeting the H. pylori glmM gene sequence as an indicator of the presence of H. pylori infection is shown in Table 3. Helicobacter pylori was detected in 24%, and 33% by histology and PCR analyses respectively, and in 41% by combining the two tests. Overall, the presence of H. pylori did not significantly differ in relation to growth pattern, localization, size, dysplasia grade of adenomas and presence of synchronous cancer.

Table 1. Clinical and pathological characteristics of the 29 gastric adenomas

	N (%)
Gender distribution	
Male	20 (69)
Female	9 (31)
Mean age (range)	68 (50–82)
Adenoma localization	
Proximal	13 (45)
Distal	16 (55)
Growth pattern	
Flat	17 (59)
Polypoid	12 (41)
Size	
<10 mm	23 (79)
≥10 mm	6 (21)
Grade of dysplasia	
Low	13 (45)
High	16 (55)
Cancer association	
Present	12 (41)
Absent	17 (59)

The mutation status of MCR of the APC gene (codons 1269–1589) and the immuhistochemical expression of COX-2 and $mPGES_1$ are reported in Table 4 and 5, respectively.

The mutation status of the MCR of the APC gene (codons 1269–1589) was assessed in 16/29 (55%) cases. Pathogenetic mutations were detected in only one case (no. 17) that carried a frameshift mutation (del G at codon 1322 with stop codon at 1413), not present in normal matched tissue (Table 4). The case corresponded to a single, small size, low-grade adenoma that was H. pylori-negative at both the histological and the molecular evaluation. No MCR sequence changes other than germline polymorphisms of not pathological significance were detected in the remaining cases: one case (no. 23) showed a novel germline G>A variant at codon 1365 (G1365S), six of 16 (38%) cases had the frequent G>A polymorphism at codon 1493 (Table 4).

The expression of COX-2 largely matched that of mPGES1. Both were overexpressed in 23 out of 29 (79%) cases regardless of histological and/or molecular evidence of H. pylori infection (Table 5). Interestingly, COX2 and mPGES1 were more frequently overexpressed in adenomas with high-grade dysplasia (15/16), >10 mm in size (five out of six) and with synchronous carcinoma (14/17). All synchronous adenocarcinomas strongly expressed both COX-2 and $mPGE2S_1$. COX-2 and $mPGES_1$ expression tended to be stronger in the surface epithelium of adenomas, in endothelial cells and in inflammatory mononuclear cells within tumor stroma (Figure 1).

The relationship between clinicopathological characteristics, COX-2 expression and H. pylori infection in single and synchronous gastric adenoma is reported in Table 6. Only the grade of adenoma dysplasia was significantly associated with the presence of synchronous gastric adenocarcinoma ($P = 0.02$).

discussion

Gastric cancer still ranks as one of the most frequent and lethal types of cancer worldwide with a survival rate at 5 years of less

Table 2. Histological findings in the surrounding stomach in patients with gastric adenomas

	Localization		Growth pattern		Size		Dysplasia grade		Synchronous cancer	
	Proximal	Distal	Flat	Polypoid	<10 mm	≥10 mm	Low	High	Present	Absent
	(n = 4)	(n = 25)	(n = 17)	(n = 12)	(n = 23)	(n = 6)	(n = 13)	(n = 16)	(n = 17)	(n = 12)
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Activity	3 (75)	13 (52)	12 (71)	4 (33)	12 (52)	2 (33)	6 (46)	10 (63)	9 (53)	5 (42)
Gastric atrophy	4 (100)	18 (72)	14 (82)	8 (66)	16 (70)	6 (100)	10 (77)	12 (75)	11 (65)	11 (92)
Intestinal Metaplasia	3 (75)	18 (72)	13 (76)	8 (66)	15 (65)	6 (100)	10 (77)	11 (69)	11 (65)	10 (83)

Table 3. H. pylori status according to histological and molecular evaluation

Diagnosis	Overall	Localization		Growth pattern		Size		Dysplasia grade		Synchronous cancer	
	(n = 29)	Proximal	Distal	Flat	Polypoid	<10 mm	>10 mm	Low	High	Present	Absent
	n (%)	(n = 4)	(n = 25)	(n = 17)	(n = 12)	(n = 23)	(n = 6)	(n = 13)	(n = 16)	(n = 17)	(n = 12)
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Histology (H)	7 (24)	1 (25)	6 (24)	4 (24)	3 (25)	6 (26)	1 (17)	5 (38)	2 (13)	6 (35)	1 (8)
Genetic (G)	8 (33)	2 (50)	6 (24)	5 (29)	3 (25)	5 (22)	3 (50)	3 (23)	5 (31)	5 (29)	3 (25)
H + G	12 (41)	2 (50)	10 (40)	6 (35)	6 (50)	8 (35)	4 (67)	6 (46)	6 (38)	9 (53)	3 (25)

Genetic, RT-PCR detection of glmM gene.

than 20% [28]. At present, prevention is likely to be the most effective means of reducing the incidence of and the mortality from this disease [29]. However, to be successful, this strategy depends upon a better understanding of the etiologic factors and the molecular mechanism underlying the carcinogenetic process.

Gastric adenoma does not appear to represent the major precursor of gastric adenocarcinoma; however, it may be considered a more specific premalignant lesion than atrophic gastritis alone [1, 30]. About 30% of the 890 cases of gastric adenoma examined endoscopically at the National Cancer Center Tokyo (Japan) were associated with cancerous changes

Table 4. Molecular analysis of MCR region (codons 1269–1589) of the APC gene in gastric adenomas

Case no.	APC (MCR region)
1	T1493T, nt 4479 (G>A), Thr→Thr
2	NA
3	T1493T, nt 4479 (G>A), Thr→Thr
4	NA
5	WT
6	T1493T, nt 4479 (G>A), Thr→Thr
7	NA
8	NA
9	NA
10	NA
11	WT
12	WT
13	WT
14	T1493T, nt 4479 (G>A), Thr→Thr
15	NA
16	T1493T, nt 4479 (G>A), Thr→Thr
17	Del G. Cod.1322 nt 3964, codon stop 1414
18	WT
19	WT
20	NA
21	T1493T, nt 4479 (G>A), Thr→Thr
22	NA
23	G1365S, nt 4479 (G>A, Gly→Ser)
24	NA
25	NA
26	NA
27	WT
28	NA
29	NA

NA, tissue not available; WT, wild type.

either within the adenoma or elsewhere in the stomach, suggesting a similar underlying pathogenesis for adenoma and carcinoma [31]. The risk of developing gastric cancer within gastric adenoma appears to increase with the size of the lesions even if relatively small adenomas can harbor infiltrating adenocarcinoma [6, 32]. Overall, gastric cancer risk ranges between 2.5% and 50% in a reported series of gastric adenomas [5, 33–38]. In our study, comparing single adenomas with adenomas synchronous to adenocarcinoma, we did not find any significant difference regarding site, size and growth pattern of adenoma. Only the grade of dysplasia was more frequent in synchronous than in single adenoma ($P <0.02$) (Table 6). In agreement with the Vienna classification [7], the grade of dysplasia may be useful to assess the potential for adenoma progression, indeed, 80% of adenomas with high-grade dysplasia progress to adenocarcinoma whereas only 15% of those with low-grade dysplasia progress to high grade or adenocarcinoma [8]. Studies from Japan have suggested an association between gastric adenomas and environmental type metaplastic atrophic gastritis and/or *H. pylori* infection [39–41]. The incidence of atrophic gastritis in patients with gastric adenoma is higher than in the general population [42]. Recently, Lee et al. [43] found extensive metaplastic atrophic gastritis in 45% of the studied population.

The high incidence of atrophic gastritis might reflect the long lasting *H. pylori* gastritis that, in turn, leads to a constant cellular stress. However, Komoto et al. [40] found that *H. pylori* infection was increased among patients with gastric adenomas but the frequency of active *H. pylori* gastritis was not different from that in controls. In our study, we found severe atrophic gastritis, intestinal metaplasia and *H. pylori* infection in 30%, 70% and 41%, respectively. Both atrophy and intestinal metaplasia were prevalently detected in adenoma with synchronous carcinoma (Table 6). It is likely that severe atrophy with loss of acid production does not favour the growth of *H. pylori* that may be no longer detectable, even though it initially induced the mucosal damage.

At present, there are no definite histological or clinical markers to identify the subgroup of adenomas progressing to adenocarcinoma. Therefore, expression profiling of genes in mucosal bioptic samples may contribute to clinical practice and may promote objective criteria for intervention, such as endoscopic mucosal resection.

In this study we aimed to see what relationship existed between clinicopathological findings of gastric adenoma, *H. pylori* infection, APC mutations and COX-2 and the down stream mPGES$_1$ expression. Our data did not reveal any significant relationship between APC germline mutation and

Table 5. COX-2 and mPGES$_1$ expression in gastric adenomas in relation to clinicopathological characteristics of gastric adenomas

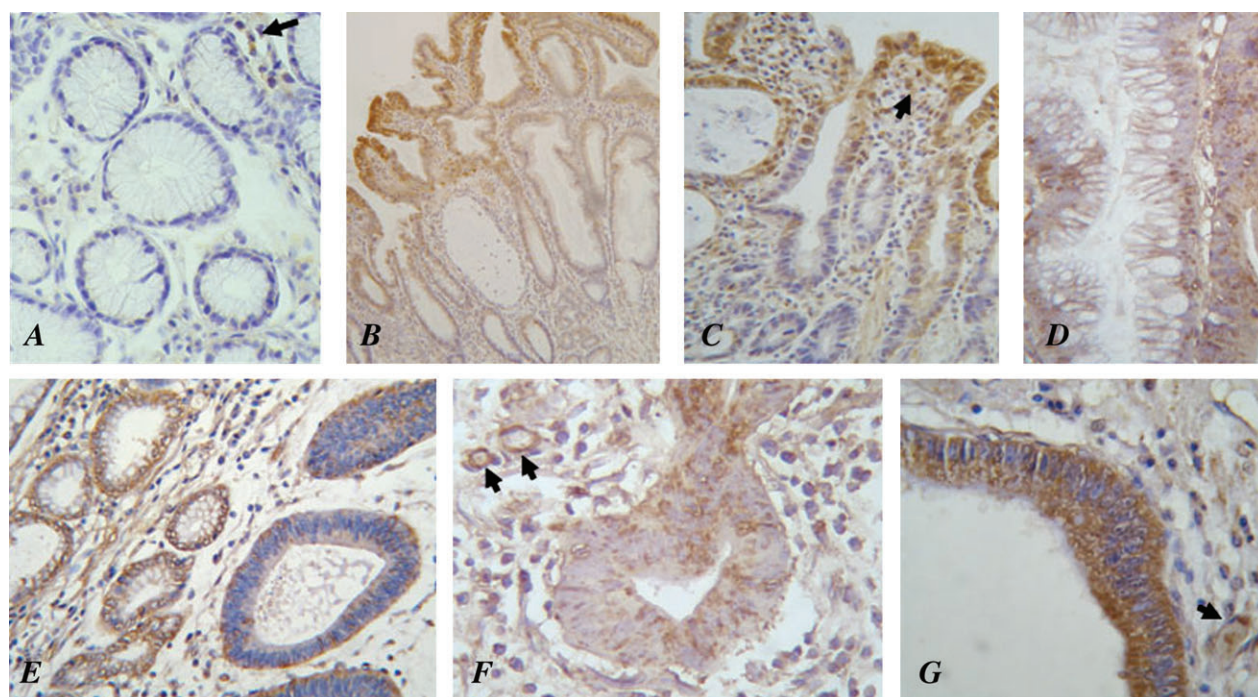| COX-2/ mPGES$_1$	Overall	Localization		Growth pattern		Size		Dysplasia grade		Synchronous cancer	
	($n = 29$)	Proximal	Distal	Flat	Polypoid	<10 mm	>10 mm	Low	High	Present	Absent
	n (%)	($n = 13$)	($n = 16$)	($n = 17$)	($n = 12$)	($n = 23$)	($n = 6$)	($n = 13$)	($n = 16$)	($n = 12$)	($n = 17$)
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Absent	6 (21)	3 (23)	3 (19)	4 (23)	2 (16)	5 (22)	1 (17)	5 (38)	1 (6)	3 (25)	3 (18)
Mild	12 (41)	4 (31)	8 (50)	7 (41)	5 (42)	11 (48)	1 (17)	6 (46)	6 (37)	5 (42)	7 (41)
Strong	11 (38)	6 (46)	5 (31)	6 (35)	5 (42)	7 (30)	4 (76)	2 (16)	9 (57)	4 (33)	7 (41)

Figure 1. COX-2 expression in normal gastric mucosa (**A**) and adenoma (**B–G**). Note the cytoplasmic positivity in the surface epithelium (**B, C**) and in the glandular cells of adenomatous (**D, E**) and periadenomatous mucosa (**E**). A strong immunostaining is also visible in the endothelial cells (**F–G**, arrows) and inflammatory infiltrate (ABC technique, original magnification A ×100; B and D ×200; C, E–G, ×400).

Table 6. Histological characteristics and COX-2 expression in relation to the presence of synchronous gastric adenocarcinoma

	Single adenoma (n = 17) n (%)	Synchronous adenoma (n = 12) n (%)	P
Proximal location	7 (41)	6 (50)	NS
Size <10 mm	12 (70)	11 (92)	NS
Flat growth pattern	8 (47)	9 (75)	NS
High grade dysplasia	6 (35)	10 (83)	0.02
Severe atrophy	3 (18)	5 (42)	NS
Intestinal metaplasia	11 (65)	10 (83)	NS
H. pylori	9 (53)	3 (25)	NS
COX2 overexpression	14 (82)	9 (75)	NS

gastric adenoma, which agrees with the observation that APC is not implicated in the malignant transformation of gastric adenoma [9].

The data concerning the expression of COX-2 and the downstream enzyme mPGES$_1$ are of particular interest. Indeed, we found both COX-2 and mPGES$_1$ overexpressed in 79% of our cases particularly in adenomas with high-grade dysplasia (15/16), >10 mm in size (five of six) and with synchronous carcinoma (14/17). COX-2 expression is one of the earliest and most frequent molecular alterations involved in gastric cancer [15, 16]. COX-2 is implicated in carcinogenesis by several mechanisms, i.e. inhibition of apoptosis, stimulation of cell proliferation, angiogenesis, mutagenic activity and signal transduction regulation [44]. Currently, while numerous reports concerning the role of COX-2 in gastric tumorigenesis

are present in the literature, only few data are available on COX-2 expression in gastric adenomas.

In conclusion, our data could suggest that COX-2 may play a role in the development and progression of gastric adenoma. Thus, COX-2 could be an attractive target in the management of gastric adenoma at major risk of cancer development.

acknowledgements

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