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Original contribution

Histologic characteristics of non-microsatellite-instable colon adenomas correlate with distinct molecular patterns[☆]

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Summary Colon carcinogenesis encompasses the stepwise accumulation of genomic aberrations correlated with the transition of aberrant crypt-adenoma-carcinoma. Recent data have revealed that, in addition to the microsatellite-instable phenotype, the chromosome instability pathway, representing four fifth of the colon carcinoma, could be involved in heterogeneous molecular alterations. Our project was aimed at determining the existence of distinct molecular subtypes in 159 non-microsatellite-instable colon polyps and their correlation with histology and dysplasia, using allelotyping, *MGMT* promoter gene methylation status, and *K-RAS* mutation analyses. Allelic imbalance, *MGMT* methylation, and *K-RAS* mutations arise in 62%, 39%, and 32% of polyps, respectively. Only 14% of polyps had no alterations. A 2-way hierarchical clustering analysis of the allelic imbalances identified subgroups of polyps according to their allelic imbalance frequency and distribution. Not only tubulovillous adenoma but also high-grade adenomas were correlated with high global allelic imbalance frequency ($P = .005$ and $P = .003$), with allelic imbalance at microsatellites targeting chromosomes 1, 6, and 9. In conclusion, the data presented in this study show that a large heterogeneity exists in the molecular patterns of alterations in precancerous colon lesions, favoring different modes of tumor initiation. Therefore, molecular alterations correlated with tubulovillous-type and high-grade dysplasia could represent targets identifying predictive factors of progression.

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1. Introduction

The prevailing model in colon carcinogenesis implies mutations and/or loss of heterozygosity of tumor suppressor genes, such as TP53 and adenomatous polyposis coli (*APC*), and mutations of oncogenes such as *K-RAS* [1]. These alterations lead to 2 classic pathways, one with chromosome instability associated with loss of heterozygosity or gene copy number gain [2], defined as the chromosomal instability (CIN) phenotype, and the other defined as the microsatellite instability (MSI) phenotype, in which DNA repair gene mutations lead to MSI and a strong index of mutations in the genome [3]. The idea that inactivation of the tumor suppressor gene *APC* serves to initiate colorectal cancers may also have important tumorigenic and pathogenic effects. The early evolution of colorectal neoplasia could occur by mechanisms other than inactivation of *APC* or related alterations that would disturb the Wnt signaling pathway [4]. Oncogenic mutations involving both *BRAF* and *K-RAS* are likely alternative initiating steps that could synergize with DNA methylation and occur within the context of serrated polyps [5,6]. Chromosome instability resulting from gene mutations involved in chromosome stability could precede *APC* mutations and be the first event initiating malignancy [7].

Among the other mechanisms that can occur in tumor initiation, alterations in DNA methylation have been reported as an early event in carcinogenesis. Inappropriate promoter hypermethylation, associated with gene silencing [8], defines the CpG island methylator phenotype, which takes part in the microsatellite and chromosome instability phenotypes [9].

Recent studies indicate that methylation abnormalities are common in precancerous colorectal lesions [10,11], in particular, methylation of the *MGMT* promoter gene [12,13]. Moreover, *MGMT* is considered a mutator gene because loss of *MGMT* expression could lead to an accumulation of mutations [14,15].

Genomic and epigenetic alterations can arise before the appearance of mutations in genes known to be at the origin of colon carcinogenesis, thus generating a growing interest in the identification of new alteration pathways responsible for the initiation of colon cancer [16,17].

Among the criteria used to stratify the risk of advanced colorectal adenoma recurrence, the number and size of adenomas are the most useful for surveillance colonoscopy [18]. However, several studies have reported the impact in risk stratification of histologic features, such as the tubulovillous or villous component and high-grade dysplasia [19-21], but to our knowledge, genomic abnormalities have not been associated with the recurrence of colorectal adenomas.

Previously, we reported that allelotyping CIN colon adenocarcinomas and their synchronous metastases revealed 3 subtypes without correlation to the allelic

imbalance (AI) frequency and the evolutionary stage [22]. These results suggested that a minimal set of alterations would be sufficient to promote tumor progression and metastasis without further alterations. These data also suggested the existence of different pathways of carcinogenesis depending on the frequency and the type of alterations. Therefore, to answer whether such molecular subtypes are observed early in carcinogenesis and to identify the molecular basis of colon cancer, a study that focused on the precancerous stage with a large number of non-MSI colon polyps was conducted. Using allelotyping, *MGMT* promoter gene methylation, and *K-RAS* mutation (K-RASm) analyses, we aimed to show the potential links between the types of molecular alterations and the histologic features of the polyps.

2. Materials and methods

2.1. Patients and tumor samples

Frozen and paraffin-embedded samples of 249 colorectal polyps, with macrodissected paired normal tissue and/or blood, were collected from 1996 to 2006 at the Biological Resources Center at the University Hospital of Strasbourg, using protocols approved by the institutional review board. Once the pathological diagnosis was established, the remaining tissue was frozen (polyps, >0.5 cm). Most of the collected polyps were obtained from synchronous colon carcinoma resections (132 of 159 polyps, resected from 101 patients). Forty polyps of MSI phenotype were excluded by allelotyping using the Bethesda microsatellite panel [23]. Fifty polyps of rectal location were also excluded. One hundred fifty-nine non-MSI colon polyps (115 males and 44 females; mean age, 68 years) from 120 patients (85 males and 35 females) were then available for further allelotyping, *MGMT* promoter gene methylation analysis, and K-RASm detection. Of the 120 patients, 93 had 1 polyp (5 resected by endoscopy and 88 during colon carcinoma resection), whereas the remaining 27 had between 2 and 6 polyps, which were obtained during carcinoma resection.

2.2. Histopathological analyses

For all cases, the histopathological type of the polyps (tubular, tubulovillous or villous adenomas, and hyperplastic polyp), sessile or conventional serrated adenomas, the degree of dysplasia (low and high grade), and the evaluation of the epithelial cell number were performed on frozen and on paraffin-embedded sections and confirmed by 2 independent experienced pathologists as described previously [24]. Table 1 summarizes the pathological features of the polyps included in this study. Twenty-nine percent of the polyps had between 50% and 30% of hyperplastic or

Table 1 Anatomic characteristics of the 159 colon polyps

	HP, n = 14	LG T, n = 93	HG T, n = 17	LG TV, n = 20	HG TV, n = 15	All polyps
Age (y)						
<60	7 (50%)	14 (15%)	3 (12%)	3 (15%)	3 (20%)	30 (19%)
≥60	7 (50%)	79 (85%)	14 (88%)	17 (85%)	12 (80%)	129 (81%)
Sex						
Men	14 (100%)	64 (69%)	12 (71%)	13 (65%)	12 (80%)	115 ^a (72%)
Women	0	29 (31%)	5 (29%)	7 (35%)	3 (20%)	44 ^a (28%)
Site						
Proximal	7 (50%)	50 (54%)	5 (29%)	10 (50%)	5 (33%)	77 (48%)
Distal	7 (50%)	43 (46%)	12 (71%)	10 (50%)	10 (67%)	82 (52%)
Size (cm)						
<1	6 (43%)	42 (45%)	2 (12%)	2 (10%)	0	49 (31%)
≥1	8 (57%)	51 (55%)	15 (88%)	18 (90%)	15 (100%)	110 (69%)
Epithelial cell count (%)						
<50	8 (57%)	30 (32%)	3 (18%)	4 (20%)	1 (7%)	46 (29%)
≥50	6 (43%)	63 (68%)	14 (82%)	16 (80%)	14 (93%)	113 (71%)
Invasive synchronous carcinoma						
Yes	12 (86%)	81 (87%)	13 (76%)	14 (70%)	12 (80%)	132 (83%)
No	2 (14%)	12 (13%)	4 (24%)	6 (30%)	3 (20%)	27 (17%)

Abbreviations: HP, hyperplastic polyp; LG T, low-grade tubular adenoma; HG T, high-grade tubular adenoma; LG TV, low-grade tubulovillous adenoma; HG TV, high-grade tubulovillous adenoma.

^a These numbers were issued from 85 male and 35 female patients.

adenomatous cells, and 71% of the polyps had greater than 50% of hyperplastic or adenomatous cells.

2.3. DNA extraction and allelotyping

DNA was extracted from paired blood, frozen polyp, and normal mucosa using the QIAamp DNA purification kit (Qiagen, Hilden, Germany). Extracted DNA was amplified by fluorescent polymerase chain reaction (PCR) as described previously [25] (see [Supporting information](#)). To detect events with potential biological impact, we used 33 microsatellites targeting 18 chromosomes corresponding to the most frequently rearranged loci found in colon cancers [26,27].

2.4. Analysis of the CpG island methylation status of the *MGMT* gene

The methylation pattern of the CpG islands in the *MGMT* gene was determined using methylation-specific PCR after the DNA (100 ng) was modified with sodium bisulfite and purified using the Epitect Bisulfite kit (Qiagen; see [Supporting information](#)).

2.5. Detection of K-RASm

To analyze K-RASm, we used the peptide nucleic acid clamping method (see [Supporting information](#)). PCR reactions were performed in the presence or absence of peptide nucleic acid using the Light cycler Fast Start DNA Master Hyb Probe kit (Roche Diagnostics). Fluorescence data were analyzed using light cycler software.

2.6. Statistical analysis

Data were analyzed using the χ^2 test and Fisher exact test.

The *P* value was considered significant when below .05. For clustering analysis, data were coded in binary form using “1” for AI and “-1” for a normal informative locus. Homozygotes were treated as missing data. Data were then clustered using a 2-way hierarchical clustering method, with uncentered correlation as similarity metrics for both genes and subjects value vectors. The average linkage was chosen as the aggregation method. Computations were run with Gene Cluster 3.0. Cluster trees were produced using Java TreeView 1.0.4 (Eisen’s Softwares, Berkeley, CA, USA).

3. Results

To identify molecular subtypes and new carcinogenesis pathways in CIN colon polyps, MSI and rectal polyps were excluded in this study.

3.1. Pathological findings

Among the 159 non-MSI consecutive colon polyps eligible for this study, 110 (69%) were tubular adenomas, and 35 (22%) were tubulovillous adenomas ([Table 1](#)). High grade represented 20% and 43% in tubular and tubulovillous adenomas, respectively, and 14 (9%) polyps were hyperplastic ([Fig. 1](#)). Villous adenomas, sessile serrated polyps, and traditional serrated adenoma were not identified. Overall, the polyp incidence was higher in men older than

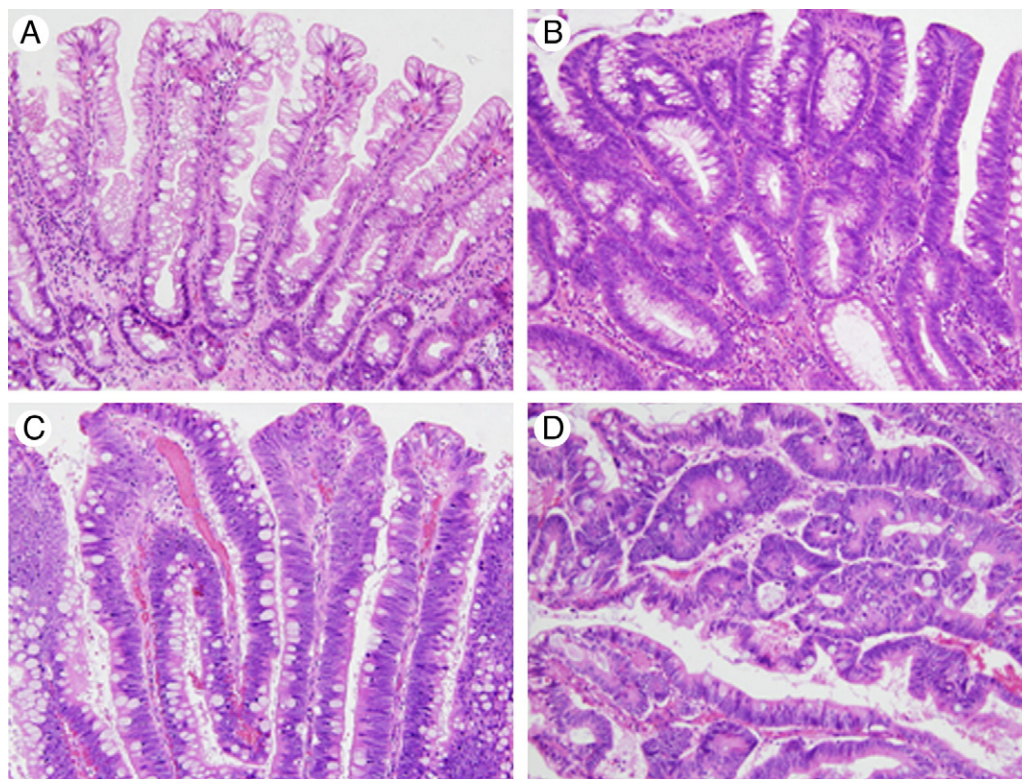


Fig. 1 Histologic examples of polyps. A, Typical morphology of hyperplastic polyp with serration at the surface and nonbranching crypts lined with proliferative cells at the base. B and C, Conventional adenoma with tubular and villous architecture, respectively. D, Nuclear atypia and architectural disorganization corresponding to high-grade adenoma (hematoxylin-eosin, original magnification $\times 20$).

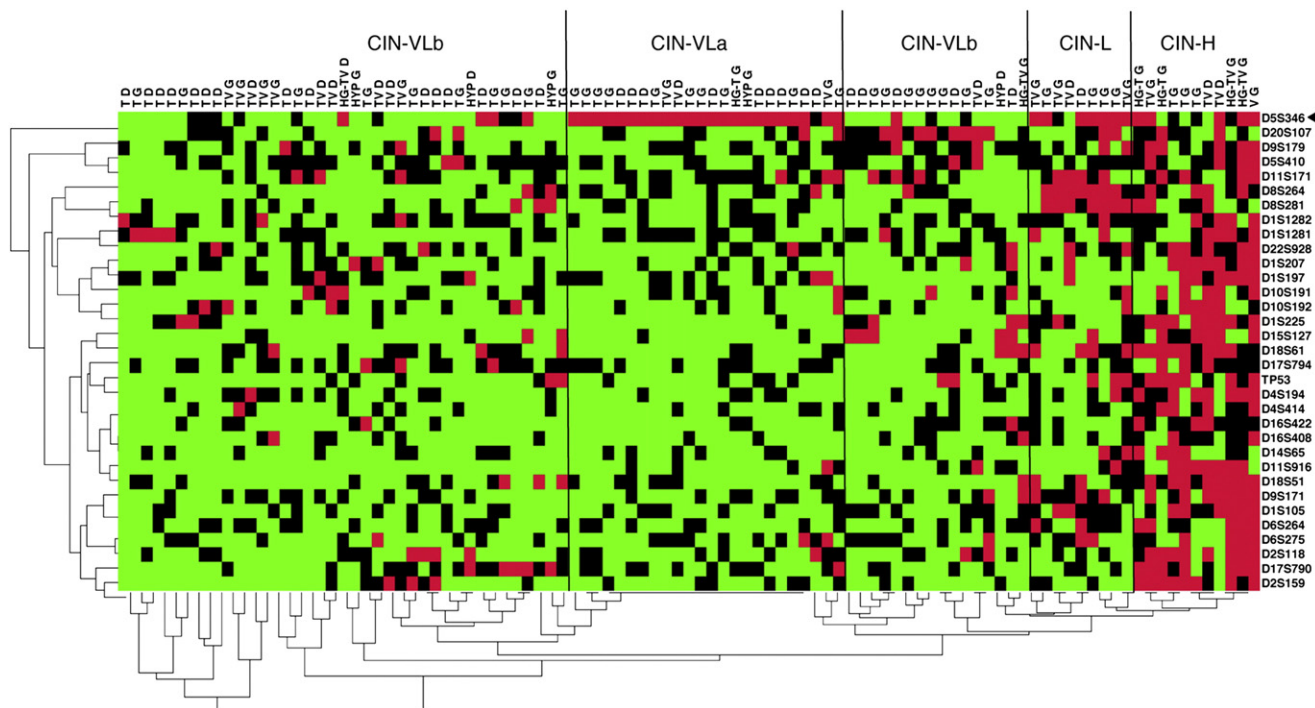


Fig. 2 Two-way hierarchical clustering of allelotyping 99 colon polyps with at least 1 AI. Red rectangles indicate AI; green rectangles, normal locus; and black rectangles, locus noninformative. Horizontal lines correspond to the microsatellites; vertical columns correspond to patients. CIN-H, CIN-L, CIN-VLa, and CIN-VLb correspond to patients with CIN with high (H), low (L), or very low (VL) frequency of alteration. Arrow head (\blacktriangleleft) indicates the *APC* locus (*D5S346*).

60 years, and no hyperplastic polyps were detected in women. High-grade adenomas of tubular or tubulovillous type were predominant in the left part of the colon, whereas hyperplastic polyps and low-grade adenomas were almost equally distributed between the right and left colon. Most polyps (83%) were synchronous with an invasive carcinoma. Among the 27 polyps removed without an invasive carcinoma, 21 (78%) were 1 cm or larger in size. Larger polyps were correlated with tubulovillous and high-grade groups ($P = .003$).

3.2. Allelotyping

Overall, 99 polyps (62%) showed at least 1 AI at an informative locus (Fig. 2). Polyps without AI ($n = 60$, or 38%) were observed in all histologic groups and grades but more frequently in hyperplastic polyps and low-grade tubular adenomas (Table 2). Among polyps larger than 1 cm, those without AI were generally right sided (34 versus 26), whereas polyps with AI were located mainly in left colon (56 versus 43), but this was not considered significant. Although the global AI frequency in the different groups of polyps showed variations (Fig. 3A), the mean global AI frequency was 3% for hyperplastic polyps (0%-15% AI), 6% for low-grade tubular (0%-52% AI), 18% for high-grade tubular (0%-56% AI), 13% for low-grade tubulovillous (0%-58% AI), and 30% for high-grade tubulovillous adenomas (0%-86% AI). High global AI frequency (46%-100% AI) was statistically associated with the tubulovillous-type ($P = .005$) and high-grade dysplasia ($P = .003$; Fig. 3B). In patients with multiple polyps (27 patients), the genomic alterations were different between the polyps for 24 of 27 patients. Interestingly, only 25% of the polyps showed an alteration at *D5S346* (microsatellite informative for *APC*

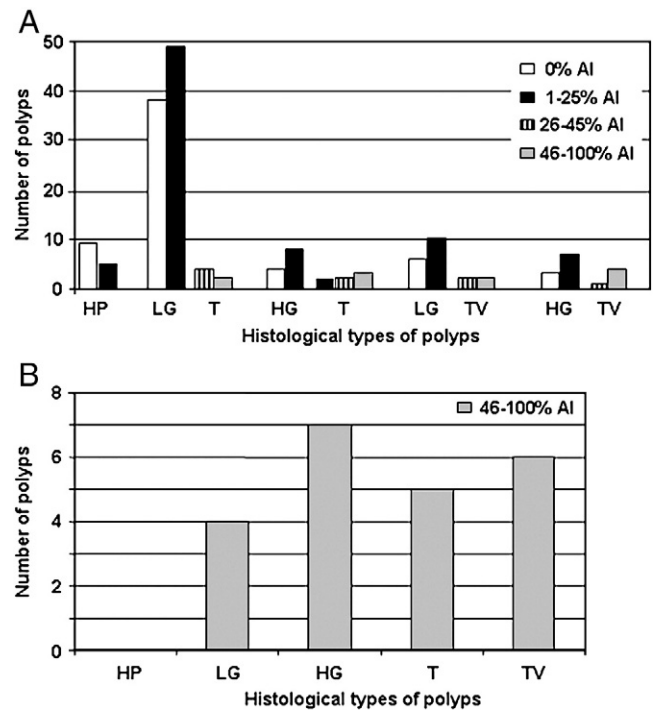


Fig. 3 Distribution of global AI frequency among the polyps. A, Distribution within the 159 polyps classified according to the different histopathological types. B, Distribution within the polyps with a high global AI frequency (46%-100% AI) classified according to the different histopathological types. HP indicates hyperplastic polyp; LG T, low-grade tubular adenoma; HG T, high-grade tubular adenoma; LG TV, low-grade tubulovillous adenoma; HG TV, high-grade tubulovillous adenoma. The global AI frequency corresponds to the number of altered loci among the microsatellites analyzed per patient.

locus). Alteration at locus *D2S159* was observed mostly in women, and alterations at loci *D14S65*, *D17S790*, and *TP53* were significantly associated to distal location (Table 3). In

Table 2 Molecular characteristics for the 159 colon polyps

	HP, n = 14	LG T, n = 93	HG T, n = 17	LG TV, n = 20	HG TV, n = 15	All polyps	Size, <1 cm	Size, ≥1 cm
AI								
Yes	5 (36%)	55 (59%)	13 (76%)	14 (70%)	12 (80%)	99 (62%)	25 (51%)	74 (67%)
No	9 (64%)	38 (41%)	4 (24%)	6 (30%)	3 (20%)	60 (38%)	24 (49%)	36 (33%)
AI only	2 (14%)	31 (33%)	6 (35%)	4 (20%)	2 (3%)	45 (28%)		
<i>MGMT</i> gene methylation								
Yes	5 (36%)	33 (36%)	8 (47%)	10 (50%)	6 (40%)	62 (39%)	16 (33%)	46 (42%)
No	9 (64%)	60 (64%)	9 (53%)	10 (50%)	9 (60%)	97 (61%)	33 (67%)	64 (58%)
Methy only	2 (14%)	15 (16%)	1 (6%)	2 (10%)	1 (7%)	21 (13%)		
K-RASm								
Yes	2 (14%)	22 (24%)	9 (53%)	8 (40%)	10 (67%)	51 (32%)	7 (14%)	44 (40%)
No	12 (86%)	71 (76%)	8 (47%)	12 (60%)	5 (33%)	108 (68%)	42 (86%)	66 (60%)
K-RASm only	0	9 (10%)	2 (12%)	2 (10%)	1 (7%)	14 (9%)		
AI at <i>D5S346</i>								
Yes	1 (8%)	23 (30%)	6 (50%)	4 (22%)	6 (46%)	40 (30%)	15 (36%)	25 (27%)
No	12 (92%)	55 (70%)	6 (50%)	14 (78%)	7 (54%)	94 (70%)	26 (64%)	68 (73%)

Abbreviations: HP, hyperplastic polyp; LG T, low-grade tubular adenoma; HG T, high-grade tubular adenoma; LG TV, low-grade tubulovillous adenoma; HG TV, high-grade tubulovillous adenoma; K-RASm, K-RAS mutation.

NOTE. The locus *D5S346* was informative for 13 polyps and indicates the *APC* gene status.

addition, alterations at *D1S225*, *D4S414*, and *D10S191* were strongly associated to be histologically tubulovillous, and alterations at *D2S138*, *D11S916*, and *D17S790* were associated with high-grade dysplasia (Table 3), whereas alterations at loci *D1S305*, *D4S394*, *D6S264*, *D6S275*, and *D9S179* showed the strongest correlation for both tubulovillous and high-grade features (Table 3).

3.3. MGMT gene methylation

To better assess the molecular status of the polyps, promoter methylation analysis of the *MGMT* gene was performed because it has been shown previously to be a potential mutator gene [14]. *MGMT* was methylated in 62

(39%) of the 159 polyps (Table 2) and was associated with alterations at loci *D3S1282* ($P = .03$) and *D9S179* ($P = .02$; Table 3). Among the 99 polyps with at least 1 AI, 39 (39%) were also found to be *MGMT* methylated. The distribution of *MGMT* methylation was homogeneous among the different histologic groups of polyps (Table 2).

3.4. K-RASms

Because the *K-RAS* gene has been suggested to initiate the adenoma-carcinoma transition, mutations at exon 1 (codons 12 and 13) were analyzed. Among the 159 polyps, 51 (32%) had K-RASm with significant correlations with the size (44 K-RASm of 110 polyps >1 cm versus 7 K-RASm of 49

Table 3 Correlations of alterations at the 33 microsatellites with clinicopathological and molecular features

MS	Chrom location	AI frequency (%)	Female vs male	Distal vs proximal	TV ^b vs T	HG ^b vs LG	MGMT methyl	K-RAS mutation
D1S197 ^a	1p33	9.6	NS	NS	$P = .005$	NS	NS	$P = .008$
D1S207 ^a	1p31.1	9.5	$P = .027$	NS	$P = .045$	NS	NS	NS
D1S225 ^a	1q42.2	9.9	NS	NS	$P = .022$	NS	NS	NS
D1S305 ^a	1q21.3	7.3	NS	NS	$P = .001$	$P = .015$	NS	NS
D2S138	2q31.1	12.3	NS	NS	NS	$P = .033$	NS	NS
D2S159	2q36.3	8.6	$P = .005$	NS	NS	NS	NS	NS
D3S1282	3q26.2	8.8	NS	NS	$P = .013$	NS	$P = .03$	$P = .007$
D3S1283	3p22-24	10.8	NS	NS	NS	NS	NS	NS
D4S394	4p16.1	7.4	NS	NS	$P = .008$	$P = .001$	NS	NS
D4S414	4q22.1	4.3	NS	NS	$P = .001$	NS	NS	NS
D5S346	5q22.2	30	NS	NS	NS	NS	NS	NS
D5S430	5p13.1	8.9	NS	NS	NS	NS	NS	NS
D6S264 ^a	6q27	6.8	NS	NS	$P = .01$	$P = .002$	NS	NS
D6S275 ^a	6q16.1	9	NS	NS	$P = .031$	$P = .005$	NS	NS
D8S264	8p23.3	12.3	NS	NS	NS	NS	NS	NS
D8S283	8p12	10.3	NS	NS	NS	NS	NS	NS
D9S171	9p21	10.7	NS	NS	NS	NS	NS	NS
D9S179 ^a	9q34.13	11.7	NS	NS	$P = .001$	$P = .002$	$P = .02$	NS
D10S191	10p13	9.8	NS	NS	$P = .034$	NS	NS	NS
D10S192	10q24.31	8	NS	NS	NS	NS	NS	NS
D11S916	11q13.4	7.6	NS	NS	NS	$P = .04$	NS	NS
D13S173	13q33.3	16.7	NS	NS	NS	NS	NS	NS
D14S65	14q32.2	4.7	NS	$P = .025$	NS	NS	NS	NS
D15S127	15q26.1	9.1	NS	NS	NS	NS	NS	NS
D16S408	16q13	3.3	NS	NS	$P = .001$	NS	NS	$P = .02$
D16S422	16q23.3	4.1	NS	NS	NS	NS	NS	NS
D17S790	17q22	11.4	NS	$P = .027$	NS	$P = .006$	NS	NS
D17S794	17q23.2	6.6	NS	NS	NS	$P = .045$	NS	NS
TP53	17p13.1	9.7	NS	$P = .04$	NS	$P = .044$	NS	NS
D18S53	18p11.21	11.7	NS	NS	$P = .015$	$P = .039$	NS	NS
D18S61	18q22.2	13.7	NS	NS	$P = .03$	$P = .034$	NS	NS
D20S107	20q12	13.8	NS	NS	NS	NS	NS	NS
D22S928	22q13.31	10.2	NS	NS	NS	NS	NS	NS

Abbreviations: MS, microsatellites; Chrom Location, chromosomal location (according to Ensembl 52); NS, not significant; TV, tubulovillous adenoma; T, tubular adenoma; HG, high-grade adenoma; LG, low-grade adenoma; MGMT methyl, *MGMT* gene methylation.

NOTE. The locus frequency corresponds to the number of altered loci among the heterozygous MS analyzed. Proximal and distal correspond to the anatomic location of the polyps. All the P values indicated are significant ($P < .05$).

^a Loci used for the hierarchical clustering as shown in Fig. 5.

^b Significant correlation in favor of TV or HG histologic types.

Table 4 Chromosomal instability, *MGMT* methylation, and K-RASm phenotypes

	A + B	A + C	B + C	A + B + C	None
HP	1 (7%)	0	0	2 (10%)	7 (30%)
LG T	12 (13%)	7 (47%)	1 (50%)	5 (25%)	13 (56.5%)
HG T	1 (6%)	1 (6.5%)	1 (50%)	5 (25%)	0
LG TV	4 (20%)	2 (13%)	0	4 (20%)	2 (8.5%)
HG TV	1 (7%)	5 (33%)	0	4 (20%)	1 (4%)
All polyps	19/159 (12%)	15/159 (9.5%)	2/159 (1.5%)	20/159 (13%)	23/159 (14.5%)

Abbreviations: HP, hyperplastic polyp; LG T, low-grade tubular adenoma; HG T, high-grade tubular adenoma; LG TV, low-grade tubulovillous adenoma; HG TV, high-grade tubulovillous adenoma. A + B, polyps with AI and *MGMT* gene methylation; A + C, polyps with AI and *K-RAS* mutation; B + C, polyps with *MGMT* gene methylation and *K-RAS* mutation; A + B + C, polyps with AI, *MGMT* gene methylation, and *K-RAS* mutation.

NOTE. Number of polyps is indicated in each clan.

polyps ≤ 1 cm, $P = .05$; Table 2), tubulovillous-type (18 K-RASm of 35 tubulovillous adenomas versus 33 K-RASm of 124 hyperplastic polyps and tubulous adenomas, $P = .01$; Table 2), and high-grade dysplasia (19 K-RASm of 32 high-grade adenomas versus 30 K-RASm of 113 low-grade adenomas, $P = .001$; Table 2). In addition, K-RASm was associated with alterations at *DIS197* ($P = .008$), *D3S1282* ($P = .007$), and *D16S408* ($P = .02$; Table 3).

3.5. Allelotyping, *MGMT* methylation, and K-RASm

We analyzed the different patterns of associations between the existence of AI, *MGMT* methylation, and K-RASm (Tables 2 and 4). Markedly, 50% of the polyps had only 1 of the 3 types of alterations (Table 2). The largest group corresponded to polyps with AI only (28%) (Table 2), integrating a subgroup with alterations at the *APC* locus (14/45, or 31%). Among the 60 polyps with no AI at the loci analyzed, 21 (13%) polyps had only *MGMT* methylation and 14 (9%) had only K-RASm (Table 2). When considering combinations of alterations (Table 4), 2 polyps had both *MGMT* methylation and K-RASm (1.5%), 15 (9.5%) had AI and K-RASms, and 20 (12.5%) had all 3 alterations. Finally, 23 polyps (14%) had no detectable alteration and were mostly represented as hyperplastic polyps (7/23, or 30%) and low-grade adenomas (13/23, or 57%), without a predominance of location (Table 4).

3.6. Clustering analyses

3.6.1. Allelotyping data

A 2-way hierarchical clustering analysis of the allelotyping data suggested the existence of 3 subgroups, according to

the AI frequency and loci distribution (Fig. 2). Most of the polyps (79/159, or 49.5%) had a global AI frequency of less than 25%, corresponding to a CIN-very low (VL) subgroup, which could be divided into 2 parts: a CIN-VLa part of 24 polyps with a mean AI frequency of 7% (3%-20%), all polyps having AI at informative locus *APC* and 11 having simultaneous K-RASm or *MGMT* methylation; and a CIN-VLb part of 55 polyps with a mean AI frequency of 8% (3%-21%), with a heterozygous distribution of AI among the different informative loci, and only 1 of these 55 polyps had an altered locus *APC*. Among these 55 polyps, only 8 had K-RASm (14.5%), 8 had only *MGMT* methylation (14.5%), and 14 had both K-RASm and *MGMT* methylation (25.5%). A second group, CIN-low (L), including 9 polyps (5.5%), had a global AI frequency between 27% and 43% (mean, 30%), with alterations mainly on chromosomes 18p, 8p, and 5p. Finally, a CIN-high (H) subgroup of 11 polyps (7%) showed strong chromosome instability, with a mean AI frequency higher than 45% (52%-86%; mean, 66%).

Proximal and distal locations were distributed equally in the CIN-VL subgroup (38 versus 41), whereas a distal location was predominant in the CIN-L (6 versus 3) and CIN-H groups (9 versus 2).

3.6.2. *MGMT* methylation, K-RASm, and *APC* locus

Clustering analysis was performed to analyze the distribution of alterations at the *APC* locus, AI, *MGMT* methylation, and K-RASm in the 99 polyps with at least 1 AI. Interestingly, the analysis of K-RASm and altered *APC* locus showed an exclusive alteration in 43 (80%) of the 54 polyps (Fig. 4). A similar exclusive alteration was observed for *MGMT* methylation and AI at the *APC* locus, with an

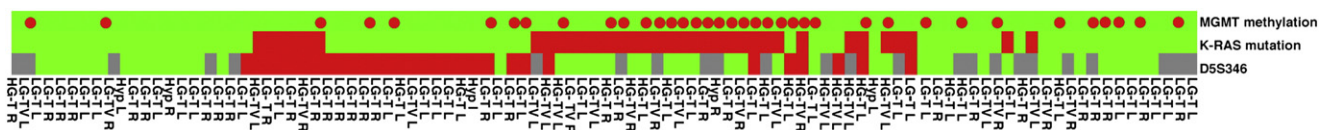


Fig. 4 *MGMT* methylation, K-RASm, and *D5S346* locus among 99 AI polyps. A 2-way hierarchical clustering was performed for the 99 polyps with at least 1 AI at an informative locus. Red rectangles indicate AI; green rectangles, normal locus; black rectangles, locus noninformative. Horizontal columns correspond to *MGMT* methylation, K-RASm, and AI for *D5S346* (informative for *APC*). Vertical columns correspond to the polyps. L indicates left adenoma; R, right adenoma.

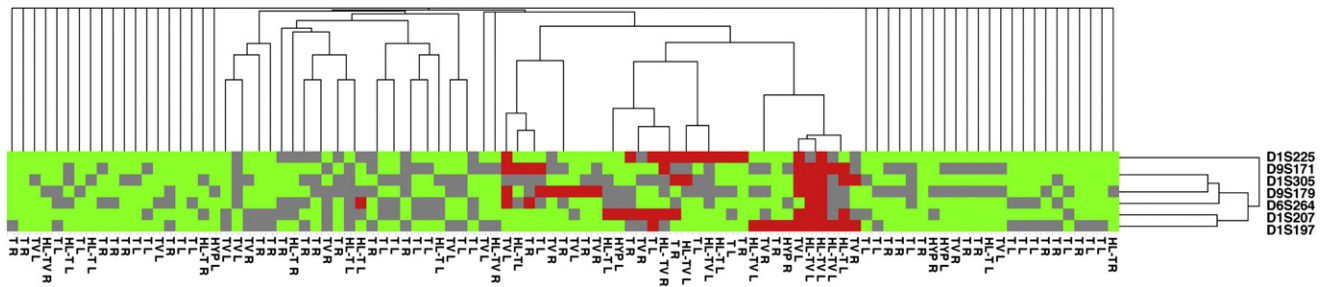


Fig. 5 Two-way hierarchical clustering of allelotyping 159 colon polyps with microsatellites targeting chromosomes 1, 6, and 9. Red rectangles indicate AI; green rectangles, normal locus; gray rectangles, locus noninformative. Vertical columns correspond to the polyps; horizontal lines correspond to the microsatellites *D1S197*, *D1S207*, *D1S225*, *D1S305*, *D6S264*, *D6S275*, and *D9S179*. HP indicates hyperplastic polyp; LG T, low-grade tubular adenoma; HG T, high-grade tubular adenoma; LG TV, low-grade tubulovillous adenoma; HG TV, high-grade tubulovillous adenoma; L, left adenoma; R, right adenoma.

exclusive alteration in 49 (85%) of the 58 polyps (Fig. 3). Finally, *MGMT* methylation and K-RASm were exclusive in 55 (74%) of the 74 polyps (Fig. 4).

3.6.3. Loci targeting chromosomes 1, 6, and 9

To show that a subset of microsatellites could represent targets for predictive factors of progression, we performed a 2-way hierarchical clustering analysis using a set of loci associated with tubulovillous adenomas and/or high-grade adenomas, because these loci are considered of higher risk for progression. Of interest, the clustering of the allelotyping data, targeting loci *D1S197*, *D1S207*, *D1S225*, *D1S305*, *D6S264*, *D6S275*, and *D9S179* for the 99 polyps, separated 60% of polyps with tubulovillous and/or high-grade features (Fig. 5).

4. Discussion

In this study, we investigated genomic alterations in a large cohort of colon polyps of a non-MSI phenotype, assessing the allelotypes of 33 microsatellites, *MGMT* promoter gene methylation, and K-RASm. We correlated these results with clinical and histologic features to identify the molecular basis of CIN colon carcinogenesis initiation. In our cohort, several distinct patterns of molecular alterations involving AI, K-RASm, and *MGMT* methylation were identified (Fig. 6), suggesting distinct pathways for the development of carcinogenesis. Interestingly, molecular alterations located on chromosomes 1, 6, and 9 were associated with the histologic tubulovillous component and high-grade dysplasia.

Our previous study focused on invasive colon cancer of the CIN phenotype [22]. Carcinomas were found to be separated into 3 subgroups, according to the global AI frequency and specific loci distribution without correlation to the clinical stage. This suggests that a subset of alterations, present at early stage, could be sufficient for metastasis. This observation is in agreement with other studies [17,28]. We therefore questioned whether such molecular subtypes could

be detected in precancerous colon lesions. For this purpose, a collection of 159 colon polyps, with established CIN status and histologic features, was analyzed. Clustering analysis identified 4 subgroups of polyps according to their AI frequency and altered loci distribution. One set (CIN-VLa) contained polyps with an altered *APC* locus and few simultaneous K-RASm, possibly corresponding to the canonical pathway [1], where the inactivation of the gene *APC* initiates the adenoma-carcinoma sequence. In subgroup CIN-VLb, polyps showed a heterogeneous distribution of AI, *MGMT* methylation, and K-RASm with missed alterations at locus *APC*, suggesting an additional pathway for tumor initiation linked to CIN. Finally, a subgroup of polyps without AI at the studied loci is characterized by *MGMT* methylation or K-RASm. In our cohort of a small number of cases without any genomic alteration, the 33 analyzed loci or *MGMT* methylation status or K-RASm or the comparative genomic hybridization array analysis available on 8 of these 23 polyps did not reveal any further specific genomic alterations (data not shown). Therefore, we hypothesize that these molecular subtypes underlie different modes of initiation of colon carcinogenesis into the non-MSI pathway,

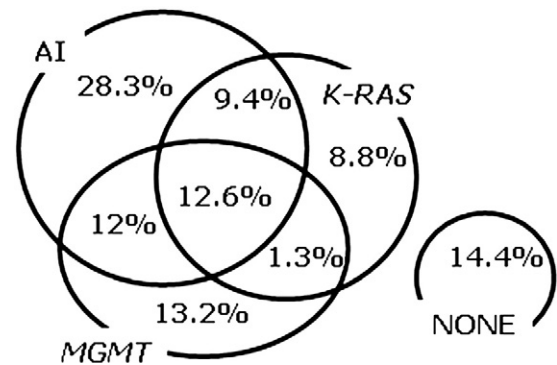


Fig. 6 Different modes of initiation of non-MSI colon carcinogenesis. There are at least 4 modes of initiation in the non-MSI pathway. *MGMT* indicates promoter methylation of *MGMT* gene; *K-RAS*, K-RASm; NONE, group free of any of the studied alterations.

in addition to the Fearon-Vogelstein model involving *APC* alteration as a primary initial event [1]. This is in agreement with the recent concept of colorectal carcinogenesis [9,29]. Apparently, colorectal cancer evolves through multiple pathways composed of many individual genetic and epigenetic alterations [9]. This multiplicity of pathways could explain the variations in tumor behavior and responsiveness to therapy [29]. Our results suggest that a potential pathway could associate K-RASm with alterations at chromosome arms 1p and 3q, but it remains to be confirmed in another cohort. In addition, the existence of a small group free of alterations reinforces other studies on colon cancer [4] in which other genes of the Wnt [30] or Notch signaling pathways [31] could initiate an adenoma.

Most colorectal carcinomas develop from preformed adenomas, but only a minority of adenomas undergo malignant transformation. As described in the literature [21,32], the risk factors for evolution of adenoma are mostly size, location, age, tubulovillous or villous histology, and the presence of high-grade dysplasia. Interestingly, in our cohort of non-MSI polyps, no villous or serrated adenoma was detected because, as expected, serrated adenomas were related to the MSI phenotype [33]. In our study, whatever alterations were considered, no significant differences between right and left location were observed. To our knowledge, studies involving colon carcinoma, which distinguished right and left locations either by large-scale transcriptome analyses or by epigenetic and genetic alterations, mostly included the MSI or CIN phenotypes [34] or addressed a small cohort [35]. By these genomic analyses, we did not observe any significant differences between right and left colon, which could be linked to the absence of MSI phenotype because the MSI phenotype is found preferentially in right-sided colon tumors [36].

We were not able to identify any independent specific locus alterations associated with size, highlighting the absence of specific genomic signatures involved in proliferation. A significant association between the sizes of the polyps, the main clinical parameter for a usual predictive factor, and the global AI frequency or K-RASm was observed in our cohort. Although K-RASm has been linked to aggressive carcinoma [37,38], no meta-analysis has confirmed this alteration as a valid prognostic factor in colorectal cancer [39].

A strong correlation was identified between the tubulovillous-type, high-grade dysplasia, and the global AI frequency. The impact of histologic features for the risk of advanced adenoma has already been demonstrated by epidemiologic studies [21,32]. To our knowledge, our study is the first that links histology and molecular alterations within a cohort of colon polyps of non-MSI status. Indeed, clustering the allelotype data on loci targeting chromosomes 1, 6, and 9 has allowed us to associate adenomas with tubulovillous histology and/or high-grade dysplasia. In the future, techniques such as comparative genomic hybridization array, single nucleotide polymorphism array, or high-

throughput sequencing will be required to identify specific genes associated with these loci.

In conclusion, genomic molecular analyses of 159 non-MSI colon polyps demonstrate a large variety of molecular alteration associations, supporting the idea that several distinct sets of alterations can be present at the precancerous stage. In addition to the subgroup related to the canonical Wnt/APC pathway, other molecular pathways for tumor initiation in precancerous colon lesions must exist. Finally, alterations in loci located on chromosomes 1, 6, and 9 are strongly correlated with tubulovillous and high-grade features. The genes that can be identified and associated with altered loci may be used to predict risk of recurrence/progression in colorectal carcinogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.humpath.2010.07.003](https://doi.org/10.1016/j.humpath.2010.07.003).

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